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THE JOURNAL OF GENERAL MICROBIOLOGY

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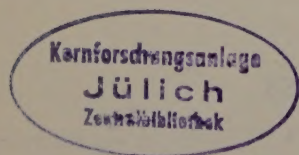
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The Microbial Metabolism of Nitro-aromatic Compounds

By R. B. CAIN

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SUMMARY: Species of *Nocardia* and *Pseudomonas*, capable of oxidatively metabolizing nitrobenzoic acids and other aromatic compounds, were isolated from soil and polluted streams. The nitro group was eliminated principally as ammonia and arylamine but some nitrite appeared. The oxidation of both *o*- and *p*-nitrobenzoic acids was adaptive and was competitively inhibited by the *m*-isomer.

Naturally occurring nitro-aromatic compounds are rare, but synthetic compounds are liberated in considerable quantities in the effluents from industrial plants, especially those manufacturing explosives (Southgate, 1948), and are increasingly used as selective herbicides. The fact that these materials do not accumulate indicates that some form of removal must occur. Recent observations of the existence of biological nitrocompounds, e.g. chloramphenicol, (Ehrlich *et al.* 1948) β -nitropropionic acid (Carter & McChesney, 1949) and the toxic nitro-compound synthesized by *Streptomyces thioluteus* (Hirata, Okuhara & Naitô, 1954) suggest the possibility of biological removal of nitrocompounds. Erikson (1941) showed that strains of *Micromonospora* were capable of utilizing trinitrophenol and trinitroresorcinol, and Moore (1949) isolated two *Nocardia* spp. which used nitrobenzene as sole source of carbon and nitrogen. No investigations of intermediate metabolic products were made in either case. Numerous reports exist of the reduction of the nitro-group by extracts of micro-organisms, e.g. *Escherichia coli* (Smith & Worrel, 1949; Saz & Slie, 1954); *Streptococcus haemolyticus* (Egami, Ebata & Sato, 1951). Simpson & Evans (1953) and Gundersen & Jensen (1956) described a different catabolism, where nitrite was liberated from nitrophenols, the former authors showing that *p*-nitrophenol was degraded by way of quinol and that catechol was an intermediate for the corresponding *o*-isomer. The present work was begun in an attempt to examine the possibility of applying biological detoxication methods to industrial effluents contaminated with nitro-aromatic compounds.

METHODS

Chemicals. All organic compounds used as substrates were recrystallized before use and were chromatographically pure. Inorganic reagents were all of A.R. quality.

Auxanography. Auxanographic techniques followed the methods of Pontecorvo (1949).

Manometry. The standard techniques for O₂ uptake were used (Umbreit, Burris & Stauffer, 1949); measurements were made at 30°.

Growth experiments. The defined medium used in most growth experiments

consisted of (g./l.): K_2HPO_4 , 0.4; KH_2PO_4 , 0.1; $MgSO_4 \cdot 7H_2O$, 0.01; aromatic compound as substrate, 1.0; trace elements solution (Barnett & Ingram, 1955) 10 ml./l. medium; adjusted to pH 7.2–7.4. Ammonium sulphate 0.5 g./l. was added when a supplementary nitrogen source was required. The medium was distributed either in tubes or in 50 ml. lots in 250 ml. Erlenmeyer flasks. On the few occasions when solid media were used, 1.5 % (w/v) New Zealand agar was added to the defined medium. All cultures were incubated at 30° unless otherwise specified.

Estimations. Nitrite was estimated by the Griess–Ilosvay method; ammonia by Nesslerization after distillation in the Markham apparatus from culture fluids, filtered to remove organisms; catechol substances by a quantitative modification of the method of Evans (1947); arylamino compounds by the Bratton–Marshall procedure as modified by Glazko, Wolf & Dill (1949) with *o*-, *m*- and *p*-aminobenzoic acids as standards; aspartic acid by decarboxylation with Chloramine-T and manometric determination of the CO_2 evolved (Cohen, 1940). Measurements of pH values were made with a Doran pH meter.

Three methods were available for the estimation of the nitrobenzoic acids: (1) colorimetrically by reduction of the nitro group by $SnCl_2$ or $TiCl_3$ and estimation as arylamine; (2) spectrophotometrically at 273 $m\mu$ and pH 7.4, (both *o*- and *p*-isomers of nitrobenzoic acid gave a rather broad but pronounced peak at this wavelength); (3) by a quantitative chromatographic method originally devised for amino acids by Fisher, Parsons & Morrison (1948). Although method (3) had a 5 % variance it was originally used because (1) would indicate all nitro-compounds, including metabolic intermediates, as amino derivatives, and in (2) the possibility of other metabolites with similar absorption maxima could not be excluded. Later work indicated that such interference did not occur and method (2) was subsequently used.

Growth was estimated turbidimetrically in a 'Unicam' model S.P. 350 D.G. colorimeter at 530 $m\mu$ using 10 mm. square glass cells, the optical density measurements being calibrated against dry-weight determinations at 110° for 2 days. Since the morphology of the isolates changed markedly according to the stage of growth, it was necessary to ensure the correspondence of morphological state in experiments with that used for constructing the turbidity/dry-weight calibration curve. To avoid interference from the colour developed in the medium during growth, the organisms were removed by centrifugation and resuspended in buffer before estimation.

Chromatography. Extracts for chromatography were de-salted either by ion-exchange methods, (Piez, Tooper & Fosdick, 1952) or more usually by precipitation of most of the inorganic material with *isopropanol*. In the latter case, there was little alteration, if any, in R_F values. Amino acids were run overnight together with authentic specimens on Whatman 20 paper in a solvent mixture of mesityl oxide + glacial acetic acid + water (50:45:15) (F. W. Moore, private communication) and were then detected with ninhydrin. This solvent mixture achieved an excellent separation of glutamic acid from aspartic acid. α -Ketoacids were run for 16 hr. as their 2:4-dinitrophenylhydrazones, after extraction with $CHCl_3$, in *tert*-amyl alcohol + *n*-propanol +

conc. NH_3 (Sp. gr. 0.88) (65:5:30) also on Whatman 20 paper, together with the authentic markers.

Staining methods. The morphology of the organisms isolated was examined by phase-contrast microscopy and in stained preparations; Gram's method and the tannic acid-crystal violet method for cell walls were used.

RESULTS

Isolation of the organisms

Enrichment cultures were prepared from samples from garden soils, manure heaps, or heavily polluted streams near Birmingham. Volumes (100 ml.) of defined medium, with the *o*-, *m*-, or *p*-isomer of nitrobenzoic acid (0.1 g.) as sole C-source, and 0.05 g. $(\text{NH}_4)_2\text{SO}_4$, were diluted to half strength with tap-water in 2 l. Carrel flasks, giving a shallow layer of solution. Samples (2–3 g.) of each source (2–3 ml. of the water samples) were added and dispersed by shaking. The cultures were kept at room temperature for 7–10 days and occasionally agitated. Four successive subcultures from these enrichments were made. Small Gram-negative and Gram-positive rods predominated in the fourth subculture. Plating upon nutrient agar, or defined medium + $(\text{NH}_4)_2\text{SO}_4$ solidified with agar, yielded well-separated colonies after incubation at 30° for 48 hr. A proportionate sample of these was subcultured into tubes of defined medium + $(\text{NH}_4)_2\text{SO}_4$ in which about 60 % of the isolates continued to grow. These latter isolates were replated and again subcultured into the appropriate medium. When the supplementary N-source was omitted, a further number failed to grow. By this means, 11 isolates of which 8 were able to utilize *p*-nitrobenzoic acid and 3 *o*-nitrobenzoic acid, were obtained. They were maintained by fortnightly subculture on nutrient agar slopes and in the defined liquid medium. No organism capable of utilizing *m*-nitrobenzoic acid was obtained during 2 years of attempted enrichment by the above methods nor by percolation techniques.

The organisms isolated

Two groups of bacteria able to utilize *p*-nitrobenzoic acid were obtained; from their morphological and biochemical reactions, the organisms comprising group CA were identified as *Nocardia erythropolis* (Gray & Thornton) Jensen, while group NC3 included pigmented and non-pigmented strains of the *Pseudomonas fluorescens* species-group.

The organisms using *o*-nitrobenzoate were colonially and morphologically very similar to those of group CA. Coccoid forms, however, were regularly produced, and nitrate was extensively reduced to nitrite by two of them (05 and 06) and weakly by the third (04). Isolates 04 and 05 most closely resemble *Nocardia opaca*, as described in *Bergey's Manual*, (1948) while 06 resembles *N. corallina*. All these species were described by Gray & Thornton (1928) as capable of metabolizing aromatic compounds.

Growth properties of the organisms

Aeration. No growth of the isolates took place in N_2 , H_2 , or CO_2 atmospheres. On defined media, with *p*-nitrobenzoate as C, N, and energy source, aerobic conditions were necessary; an increased CO_2 tension was not favourable. For work with washed suspensions, large amounts of organisms were grown in 15–20 l. of medium at 30° in a 25 l. aspirator, aerated by forcing sterile air through the medium with a compressor. Between 24 and 30 g. of wet organism of high activity were thus consistently obtained. Contamination was rare. Large trays covered with solid medium, a method used with considerable success in this department for cultivating *Serratia* spp. (Cartwright, 1955), did not give much growth with the present organisms and there was no improvement when the agar was well-washed.

Effect of nitrobenzoate concentration. Growth of the *Nocardia* isolates at different concentrations of *o*- or *p*-nitrobenzoate occurred over the range 0.05–1.0 % (w/v), but no growth of the two pseudomonad isolates occurred on the *p*-isomer above 0.35 %. At higher concentrations, there was an increased lag before growth became visible and the yield of organism decreased. The optimum concentration of *p*-nitrobenzoate for *Nocardia erythropolis* was 0.25 %; for *N. opaca* the optimum *o*-nitrobenzoate concentration was somewhat lower.

Effect of temperature. On the defined medium, the optimum temperature range for all the *Nocardia* isolates was 25 – 30° ; and for the pseudomonads 20 – 22° . The *Nocardias* grew over the range 15 – 30° ; the pseudomonads, however grew slowly at 4° and at 37° but not higher.

Effect of pH value. Medium containing either *p*-nitrobenzoate or *o*-nitrobenzoate was adjusted with HCl or KOH to initial pH values from 3.6 to 9.3 and sterilized by Seitz filtration. No growth of *Nocardia erythropolis* was visible on *p*-nitrobenzoate medium below pH 6.4 or of the pseudomonads below pH 6.1 at 14 days. Optimal growth of both forms occurred at pH 7.3–7.6 in 3–5 days. The *Nocardia* isolates using *o*-nitrobenzoate also showed pH optima in the alkaline side of neutrality.

Effect of growth factors. Yeast extract, casein hydrolysate, or 'Lab-Lemco', each markedly stimulated the growth of all isolates on the defined medium. Yeast extract was therefore regularly incorporated in the medium for growing large batches of organisms in the aspirator, because, although it decreased the selectivity of the medium, it increased the yield without appreciably altering the activity shown by the organism in subsequent manometric studies. Auxanographic tests showed that the effect of adding yeast extract could not be entirely attributed to any one or combination of 27 amino acids, purines, pyrimidines, or vitamins. Slight growth stimulation was noted in some cases.

Substrates for growth. Tests of the ability of the isolates to utilize a wide range of aromatic compounds were made in the defined medium + $(NH_4)_2SO_4$ with the test compound at 0.1 % (w/v) inoculated with 0.2 ml. of a 3-day culture of *Nocardia erythropolis* or *N. opaca* grown with *p*- or *o*-nitrobenzoate, respectively. Inability to utilize a substrate was recorded when there was no

visible growth after 7 days. Table 1 shows the results obtained by a representative isolate of both species. Both isolates utilized as sole C source, acetate, pyruvate, malate, succinate, fumarate and citrate; formate supported only weak growth; β -ketoadipate was not utilized by either isolate. Most of the mono- and dihydric phenols tested, whether or not nitrated, did not support growth at 0.02 % (w/v); at 0.05 %, they inhibited growth in the nitrobenzoate substrates.

Table 1. *Substances tested for ability to support growth of Nocardia erythropolis and N. opaca*

Substrate	% (w/v)	Degree of growth at 7 days	
		<i>N. erythropolis</i>	<i>N. opaca</i>
<i>p</i> -Nitrobenzoic acid	0.1	++++	—
<i>o</i> -Nitrobenzoic acid	0.1	—	++++
<i>m</i> -Nitrobenzoic acid	0.1	—	—
<i>o</i> -, <i>m</i> -, or <i>p</i> -Nitrophenols	0.025	—	—
2-, 3-Hydroxy-4-nitrobenzoic acids	0.1	—	—
<i>p</i> -Hydroxybenzoic acid	0.1	++++	++
<i>p</i> -Toluic acid	0.1	+++	nr
<i>o</i> -Hydroxybenzoic acid	0.1	+	++
Protocatechuic acid	0.1	++++	nr
<i>p</i> -Aminobenzoic acid	0.1	±	—
2:4-Dinitrobenzoic acid	0.1	+	+
Quinic acid	0.1	++++	++++
2:4; 2:5; or 2:6-Dihydroxybenzoic acids	0.1	±	nr
<i>p</i> -Nitrobenzaldehyde	0.02	±	—
Phenol	0.02	+++	++
Catechol	0.02	—	—
Quinol	0.02	—	—
Resorcinol	0.02	—	—
Benzoic acid	0.1	++++	++++
<i>p</i> -Hydroxyphenylacetic acid	0.1	++	nr
<i>p</i> -Hydroxyphenyllactic acid	0.1	++	nr

Observations made at 7 days; — = no growth; ±, +, ++, +++, etc. = degrees of growth estimated visually. nr = not recorded.

Nitrogen metabolism of the nitrobenzoic acids

The finding that *o*- and *p*-nitrobenzoate could provide the sole source of nitrogen as well as of carbon for *Nocardia opaca* or *N. erythropolis* prompted investigation into the fate of the nitrogen atom in the earlier stages of growth. The only significant recovery of total available nitrogen from the defined growth media with *p*-nitrobenzoate, was made as NH_3 and arylamine, although traces of nitrite were observed (Fig. 1). An interesting feature was the rapid initial ammonia production which accounted for approximately one-quarter of the total nitrogen content of the medium. This ammonia production together with arylamine (both calculated at the maxima) usually accounted for up to one-third of the total fixed nitrogen. The values of these metabolites when *o*-nitrobenzoate was present were generally somewhat lower.

The fate of the remaining two-thirds of the original total nitrogen is obscure, but is certainly not all accounted for by an increase in bacterial-N, although it is not unreasonable to assume that some nitro-N is directly 'assimilated' (Clifton, 1946). A micro-Kjeldahl estimate of the unidentified combined nitrogen in the medium accounted for a further one-quarter of the original total *p*-nitrobenzoate-N.

The pH of the medium, which increased steadily during growth to a maximum of about 8.8 before falling, could be correlated with changes in the ammonia concentration. This high initial ammonia production was independent of the initial pH of the medium. The observation that the ammonia concentration diminished after reaching a maximum suggested that it was probably being utilized by the organism. Aspartase activity was investigated with cell-free extracts, prepared by alumina grinding (McIlwain, 1948) of fresh or freeze-dried organisms, grown on *o*- or *p*-nitrobenzoate. Incubation of fumarate and $(\text{NH}_4)_2\text{SO}_4$ with cell-free extracts in an atmosphere of O_2 -free nitrogen indicated the formation of aspartic acid, detected chromatographically in the mesityl oxide solvent. Quantitative estimations of aspartase activity (Table 2) were made, allowing for the interference due to the ammonia present, which was independently estimated. Parallel controls were run without added ammonia to correct for the slight endogenous production of this metabolite.

Table 2. *The aspartase activity of extracts of Nocardia erythropolis*

Each Warburg Flask contained: 2.5 ml. 0.05M-tris, pH 7.2; 0.5 ml. extract ($\cong 4.8$ mg. protein); 5 μmole Na fumarate; 5 μmole $(\text{NH}_4)_2\text{SO}_4$ (absent in the control flasks) in a total volume of 4.0 ml. Gas phase, O_2 -free nitrogen; temp. 30° ; shake speed, 120/min. Experimental and control flasks were removed at the times indicated, deproteinized with 0.5 ml. 10% (w/v) trichloroacetic acid, and analysed for NH_3 and aspartate.

Time (min.)	NH_3 (μmole)	Aspartic acid (μmole)
0	5.00	0
30	4.69	0.04
60	4.83	0.40
90	4.83	0.45
120	4.65	0.73
160	4.15	0.45

Qualitative chromatographic experiments also indicated significant transaminase activity in these crude extracts. Incubation, under nitrogen, of aspartic and α -ketoglutaric acids yielded significant amounts of glutamic and oxaloacetic acids. Control experiments without either aspartate or α -ketoglutarate yielded traces of glutamate, but no oxaloacetate was found.

Table 3 shows the relative arylamine production from the three isomers of nitrobenzoic acid by organisms grown on *p*-nitrobenzoate and *o*-nitrobenzoate.

Relationship between p-nitrobenzoate concentration and arylamine production by Nocardia erythropolis. An investigation was made to determine whether reduction of the aryl-nitro group by the organism used here could take place. Excess of *p*-aminobenzoate was first found to exert no inhibitory effects

Table 3. Arylamine production from the nitrobenzoic acids by extracts of *Nocardia erythropolis* and *N. opaca*

Each Warburg flask contained: 3.0 ml. 0.05 M-tris buffer, pH 7.2; cell extract (3.3 mg. protein for *Nocardia erythropolis*, 2.8 mg. protein for *N. opaca*); 5 μ mole substrate (*p*- and *o*-nitrobenzoate, respectively). Total volume 4.0 ml. Gas phase, air; temp. 30°; incubation time 16 hr.

Substrate	Arylamine (μ g./ml.) Extracts of cells grown on	
	<i>o</i> -Nitrobenzoate	<i>p</i> -Nitrobenzoate
<i>o</i> -Nitrobenzoate	7.0	1.5
<i>m</i> -Nitrobenzoate	2.8	3.5
<i>p</i> -Nitrobenzoate	2.2	4.7
None	0.1	0.4

towards *Nocardia erythropolis*, grown on *p*-nitrobenzoate. Duplicate 250 ml. Erlenmeyer flasks, containing *p*-nitrobenzoate 0.0–0.5% (w/v) in the defined medium (50 ml.) were inoculated and estimations made daily after visible growth had appeared. Although *p*-nitrobenzoate concentration and maximum arylamine production were related up to 0.3% *p*-nitrobenzoate, above this value, the correlation was no longer linear, due at least in part to the weaker growth at higher concentrations of *p*-nitrobenzoate. The arylamine production/organism, however, was directly related to the nitrobenzoate concentration over the whole range tested (Fig. 2). Even so, the maximum production of *c.* 40 μ g.

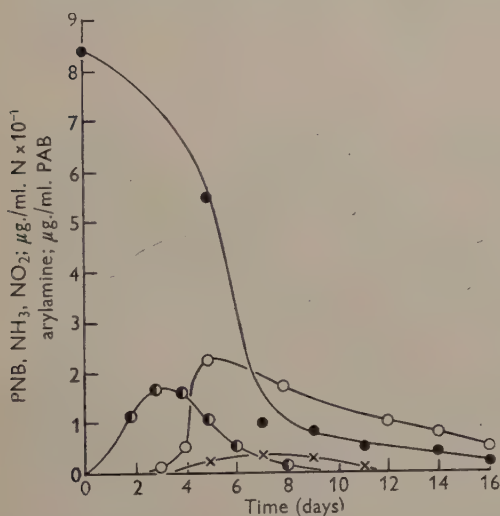


Fig. 1

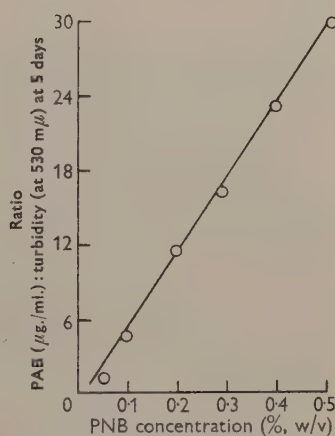


Fig. 2

Fig. 1. The nitrogen metabolism of *p*-nitrobenzoic acid by *Nocardia erythropolis*. The medium originally contained 0.1% (w/v) *p*-nitrobenzoate ($\equiv 84 \mu$ g. nitrobenzoate nitrogen/ml.). Temp. $30 \pm 2^\circ$; aerated; pH at inoculation 6.9. ●—●, *p*-nitrobenzoate remaining; ●—●, arylamine formed; ○—○, ammonia formed; ×—×, nitrite formed.

Fig. 2. Relationship between maximal arylamine production/organism and *p*-nitrobenzoate concentration. Arylamine (determined as *p*-aminobenzoate) and turbidity estimated daily until maximum value of the former reached. Medium containing varying *p*-nitrobenzoate concentrations inoculated with a washed suspension (in sterile saline) of *Nocardia erythropolis*. Temp. $30 \pm 2^\circ$; stationary cultures; pH 7.4.

arylamine/ml. ($3 \times 10^{-4} M$ as *p*-aminobenzoate) was very small compared with the initial *p*-nitrobenzoate concentration ($3 \times 10^{-2} M$) and would be unlikely to contribute to detoxication.

The adaptive nature of nitrobenzoate metabolism by Nocardia spp.

The possible adaptive nature of *p*-nitrobenzoate degradation was suggested by lag periods of 2–3 days, which were observed after inoculating defined medium with organisms grown in glucose asparagine medium or in nutrient broth. An inoculum of organism grown on *p*-nitrobenzoate medium yielded visible growth in 18–24 hr. Further investigations were made with washed suspensions, by the technique of Stanier (1947). Unadapted organisms were grown in half-strength nutrient broth containing 5 ml. of 10% (w/v) solution of Tween 80/l. to obtain diffuse growth; without Tween 80 a heavy pellicle formed. The high endogenous respiration of organisms grown on *p*-nitrobenzoate or on broth was decreased by storage overnight at 4° as a thick suspension.

Organisms grown on *p*-nitrobenzoate took up O_2 immediately upon the addition of *p*-nitrobenzoate, whereas organisms grown in broth or glucose asparagine medium showed lags of 90–120 min. before the O_2 -uptake exceeded the endogenous value. Addition of 2:4-dinitrophenol ($M/6000$) prevented this adaptation with broth-grown organisms but with pre-adapted cells this concentration of 2:4-dinitrophenol increased the rate of O_2 uptake, possibly because of interference with 'oxidative assimilation' (Clifton, 1946), see Fig. 3. Similar results were obtained for adaptation to the *o*-isomer by *Nocardia opaca*.

No evidence was found to suggest that bacteria isolated on one isomer of nitrobenzoic acid adapted to the other or to benzoic acid, as might be expected. *Nocardia erythropolis* grown in broth showed lag times of 90–100 min. with *p*-nitrobenzoate; no adaptation was shown towards the *o*-isomer at 270 min. and the *m*-isomer usually depressed the endogenous respiration (Fig. 4). With *N. opaca* the corresponding lag times were > 270 and 40 min., respectively; the *m*-isomer again decreased the endogenous respiration.

Wide differences in organism and substrate concentration were without appreciable effect on the rate of adaptive enzyme formation in *Nocardia erythropolis* and *Pseudomonas fluorescens*; but increase in pH from 6.4 to 7.6 reduced the adaptation time to *p*-nitrobenzoate from over 5 hr. to about 100 min. with *N. erythropolis*.

The specificity of the adaptive response in Nocardia erythropolis

Nocardia erythropolis was grown on a range of analogues of *p*-nitrobenzoate in which either the *p*-group and the carboxyl group, or the carboxyl alone, was altered. Because several of the analogues were carbon sources only, a specific N-source, casein hydrolysate, 'Vitamin free' (Allen & Hanbury) was incorporated in the defined medium to 0.1% (w/v). No yeast extract was present in these media, (cf. Mirick, 1943). At the same time, the concentration of C-source was reduced to 0.05% since some were toxic.

The medium was adjusted to pH 7.4–7.6 and inoculated with a sterile washed suspension of *Nocardia erythropolis* grown on nutrient agar. After harvesting and washing the growth after 5 days at 30°, the Q_{O_2} of these organisms

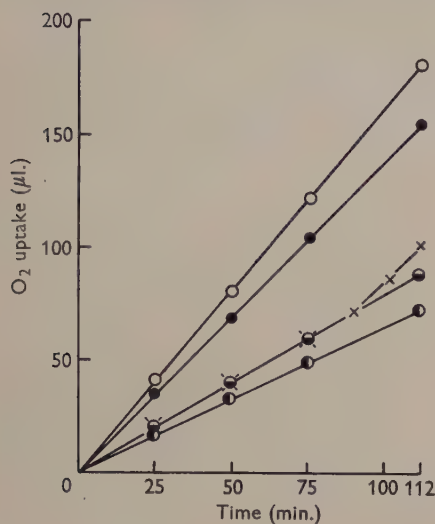


Fig. 3

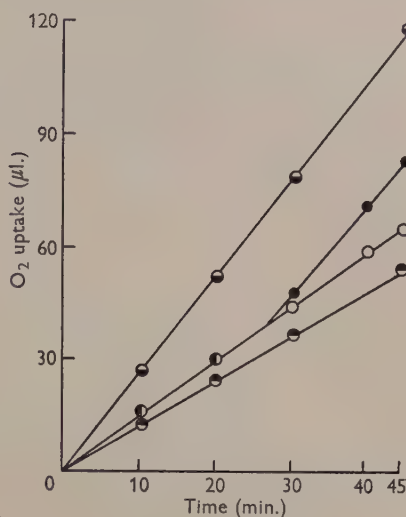


Fig. 4

Fig. 3. The adaptive nature of *p*-nitrobenzoate oxidation. Each Warburg flask contained: 0.5 ml. of a 0.05 % (w/v) solution of substrate in the side-arm; 1.5 ml. cell suspension (≈ 15 mg. dry wt.); 0.2 ml. 20 % KOH; 0.3 ml. 0.0017 M-2,4-dinitrophenol where required, and phosphate buffer, pH 7.0 to give a final volume of 3.0 ml. Washed suspensions of *Nocardia erythropolis* grown on *p*-nitrobenzoate or nutrient broth + Tween 80 were standardized turbidimetrically. Gas phase, air; temp. 30°; shake speed, 120/min. ○—○, *p*-nitrobenzoate-grown cells; ●—●, *p*-nitrobenzoate + 0.00017 M-dinitrophenol. ●—●, *p*-nitrobenzoate-grown cells; ×—×, broth-grown; *p*-nitrobenzoate. ●—●, endogenous respiration (same for both *p*-nitrobenzoate- and broth-grown cells). ●—●, broth grown; *p*-nitrobenzoate + 0.00017 M-dinitrophenol.

Fig. 4. Adaptive responses of *Nocardia erythropolis* to the nitrobenzoates: conditions as for Fig. 3, but phosphate buffer, pH 7.4 was used. ●—●, *p*-nitrobenzoate-grown cells; *p*-nitrobenzoate as substrate. ●—●, glucose-grown cells; *p*-nitrobenzoate as substrate. ○—○, glucose-grown cells; endogenous, benzoate, *o*-nitrobenzoate. ●—●, glucose-grown cells; *m*-nitrobenzoate.

Table 4. The effect of analogues on the pre-stimulation of the *p*-nitrobenzoate-oxidizing system

O_2 uptake was measured in the Warburg apparatus from 10 to 40 min. (In each case the uptakes during this period were linear). Each flask contained: suspension 2.0 ml. in buffer, pH 7.4; 20 % KOH 0.2 ml.; PNB ($\approx 2 \mu\text{mole}$) 0.5 ml.; 0.067 M-phosphate buffer, pH 7.4, to give a final volume of 3.0 ml. All O_2 uptakes were corrected for endogenous respiration.

Analogue	Q_{O_2} on PNB
<i>p</i> -Nitrobenzoic acid	4.7
Benzoic acid	4.6
<i>p</i> -Bromobenzoic acid	3.4
Sulphanilic acid	1.2
<i>p</i> -Toluic acid	0.7
<i>p</i> -Hydroxyphenylacetic acid	0.6
<i>p</i> -Hydroxybenzoic acid	0.3

was determined with *p*-nitrobenzoate as the substrate. Q_{O_2} values for *p*-nitrobenzoate oxidation by organisms grown on the defined medium without casein hydrolysate or other supplementary N-source, rarely exceeded 7.0, so that these values are not exceptionally low. *p*-Aminobenzoate, *p*-chlorobenzoate and *p*-hydroxybenzaldehyde did not induce the *p*-nitrobenzoate-oxidizing system. The pronounced inducing capacity of benzoate suggests a similar pathway of metabolism for this compound or, alternatively, that a common inducible precursor can give rise to enzymes oxidizing either benzoate or *p*-nitrobenzoate.

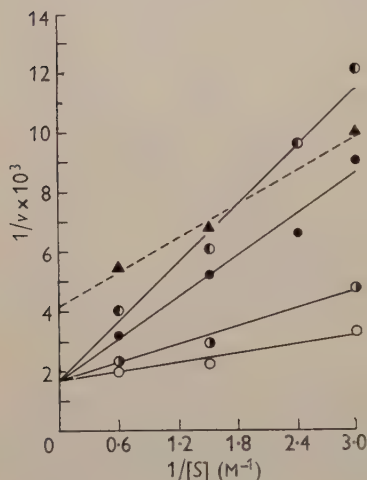


Fig. 5

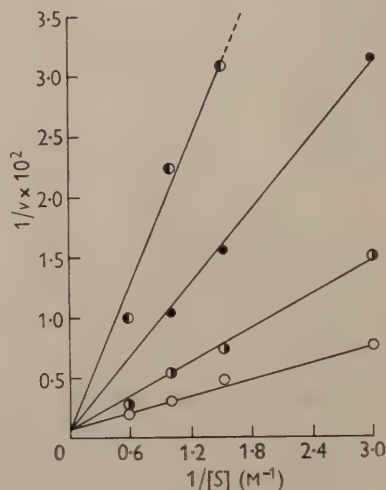


Fig. 6

Fig. 5. Competitive inhibition of *o*-nitrobenzoate oxidation by *m*-nitrobenzoate. Each flask contained: 0.2 ml. KOH; 0.5 ml. washed suspension of *Nocardia opaca* (≈ 20.3 mg. dry wt.); substrate 1–5 μ mole; inhibitor 0–2 μ mole; 0.067 M-phosphate buffer, pH 7.0, to total volume 3.0 ml. The inhibitor was tipped at zero time and the flasks shaken 3 min., the substrate was then tipped and the oxygen uptake/hr. measured for the period 5–20 min. O_2 uptakes/hr. were corrected for endogenous respiration and the low rate of oxidation of the *m*-isomer, which did not vary appreciably with concentration. The rate of O_2 uptake was used as the velocity constant of the oxidation. \bigcirc — \bigcirc , no inhibitor; \bullet — \bullet , 0.1 μ mole *m*-nitrobenzoate; \bullet — \bullet , 0.5 μ mole *m*-nitrobenzoate; \bullet — \bullet , 1.0 μ mole *m*-nitrobenzoate; \blacktriangle — \blacktriangle , 2.0 μ mole *m*-nitrobenzoate.

Fig. 6. Competitive inhibition of *p*-nitrobenzoate oxidation by *m*-nitrobenzoate. Each flask contained: 0.2 ml. KOH; 0.5 ml. washed suspension of *Nocardia erythropolis* (≈ 18.6 mg. dry wt.); substrate, 1–5 μ mole; inhibitor, 0.5–4 μ mole; 0.067 M-phosphate buffer, pH 7.0, to a total volume of 3 ml. The technique was altered slightly because cells used were found not to exhibit their maximum Q_{O_2} till about 20 min. after tipping the substrate. O_2 uptakes were thus measured over the period 25–40 min. after tipping the inhibitor. All other factors as for Fig. 5. \bigcirc — \bigcirc , no inhibitor and 0.1 μ mole *m*-nitrobenzoic acid; \bullet — \bullet , 1.0 μ mole *m*-nitrobenzoic acid; \bullet — \bullet , 2.0 μ mole *m*-nitrobenzoic acid; \bullet — \bullet , 4 μ mole *m*-nitrobenzoic acid.

Inhibition studies with the nitrobenzoic acids

The failure to induce *Nocardia erythropolis* to oxidize or grow on the *o*-isomer and *N. opaca* to oxidize or grow on the *p*-isomer of nitrobenzoic acid, together with low rates of oxidation of *m*-nitrobenzoate by organisms grown on either

p- or *o*-isomers, suggested that inhibitory effects between isomers might occur when they were present together. Such effects were tested for manometrically. Figure 5 shows a reciprocal plot (Lineweaver & Burk, 1934) of the results obtained with *N. opaca* (grown on *o*-nitrobenzoate) in the presence of *m*-nitrobenzoate. For *o*-nitrobenzoate oxidation, the inhibition by the *m*-isomer was competitive over the range 0.1–1.0 μ mole. The *m*-isomer also competitively inhibited the oxidation of *p*-nitrobenzoate by *N. erythropolis* over the range 1–4 μ mole (Fig. 6). The extent of inhibition is shown in Table 5. Calculation of K_s and K_i from Fig. 5 and 6 showed that for *o*-nitrobenzoate the affinity of the natural substrate for enzyme was about $5\frac{1}{2}$ times that of the inhibitor for enzyme when these were in similar concentrations. For the *p*-isomer this substrate:inhibitor affinity ratio was nearly 14:1.

Table 5. *The extent of inhibition by m-nitrobenzoate and nitrophenols of the oxidation of o- and p-nitrobenzoates by Nocardia opaca and N. erythropolis*

The percentage inhibition is calculated from the rate of oxygen uptake/hour for organisms in the absence and presence of different concentrations of *m*-nitrobenzoate within the range causing competitive inhibition. The figures for *m*-nitrobenzoate are deduced from the competitive inhibition curves of Figs. 5 and 6.

Concentration of inhibitor (μ mole/3 ml.) <i>m</i> -Nitrobenzoic acid	<i>o</i> -Nitrobenzoate oxidation by <i>N. opaca</i>			<i>p</i> -Nitrobenzoate oxidation by <i>N. erythropolis</i>		
	Concentration of substrate (μ mole/3 ml.)					
	1.0	2.0	5.0	1.0	2.0	5.0
	% inhibition					
0.1	33	25	13	0	0	0
0.5	66	54	38	—	—	—
1.0	74	64	50	49	33	49
2.0	—	—	—	75	68	61
4.0	—	—	—	86*	83	87
<i>p</i> -Nitrophenol 3×10^{-2}	—	79	—	—	66	—
<i>o</i> -Nitrophenol 3×10^{-2}	—	62	—	—	71	—

* Approximate only. — = not recorded

There was no inhibition of the oxidation of *p*-nitrobenzoate by the *o*-isomer, or by other *o*-substituted compounds, e.g. anthranilic and salicylic acids, or of the oxidation of *o*-nitrobenzoate by the *p*-isomer and comparable *p*-substituted compounds.

DISCUSSION

Low but significant rates of *m*-nitrobenzoate oxidation were found with organisms grown on either *p*-nitrobenzoate or *o*-nitrobenzoate, whereas the former were never found to oxidize *o*-nitrobenzoate, nor did the latter oxidize *p*-nitrobenzoate. In addition, extracts of alumina-ground organisms grown on *p*-nitrobenzoate yielded significant quantities of arylamine only from *p*- and *m*-nitrobenzoates (Table 3). Similar extracts of organisms grown on *o*-nitrobenzoate reduced principally the *o*- and *m*-isomers.

These results are explicable on the hypothesis that the spatial configuration of the *m*-isomer permits the formation of an enzyme-substrate complex with

enzymes which have been induced by the presence of either *o*- or *p*-isomers, assuming that the nitro and carboxyl groups are the reactive ones. The same hypothesis can explain the observed competitive inhibition by *m*-nitrobenzoic acid of both the *p*-nitrobenzoate oxidation by organisms grown on *p*-nitrobenzoic acid, and *o*-nitrobenzoate oxidation by cells grown on *o*-nitrobenzoic acid. It also accounts for the observed absence of inter-inhibitory effects between the *o*- and *p*-isomers. The effect can thus be ascribed to a form of 'biological steric hindrance' by the nitro-group, similar to the well-known examples of inhibition among growth-factor analogues. Why the range of inhibitor concentration required for competitive inhibition of oxidation of the *p*-isomer is so much greater than that required for similar inhibition of *o*-nitrobenzoate oxidation is not yet clear, but the explanation probably lies in a comparison of the nature of the early metabolic products (as yet undetermined) of the *o*-isomer and those of the *p*-isomer.

The finding that nitrobenzoates would support good growth only under alkaline conditions suggests that only in the dissociated form is the substrate assimilated. Krah1 & Clowes (1938) found that the inhibitory effect of nitro-substituted aromatic compounds on *Arbacia* eggs was due to penetration of the cell membrane by the undissociated molecule. Gundersen and Jensen (1956), using a variant of *Corynebacterium simplex*, found that alkaline conditions, pH 7.3-8.5, were essential for the rapid dissimilation of several mono- and dinitrophenols by this organism. The results of Simon & Beevers (1952) showed that the toxicity of weak acids was due to the undissociated molecule. At values below the pK of the acid, the pH value had no effect, but above this value, the concentration of the undissociated molecules and hence the toxicity, fell rapidly. At higher pH values, therefore, such substrates may be metabolized.

The pK values for *o*- and *p*-nitrobenzoic acids are 4.23 and 5.62, respectively, so that, at the pH at which optimal growth of *Nocardia opaca* and *N. erythropolis* occurs on these substrates, pH 7.3-7.6, the concentration of undissociated acid is very low. The production of ammonia, which further raises the pH value, tends to decrease this concentration even more.

The disappearance of nitro-aromatic compounds under natural conditions would thus appear to depend largely on the pH value of the environment. Toxic effects due to the accumulation of nitro-aromatic compounds are consequently more likely to occur in acid soils than in alkaline conditions. It is suggested, therefore, that it is in acid soils, where they are stable to microbiological attack for longer periods, that nitro-aromatic compounds will be most successful as herbicides.

My sincere thanks are due to Dr N. J. Cartwright for his continued encouragement and technical advice during this work. I should like to express my appreciation both to him and to Dr K. A. Bisset for their advice on presentation of the manuscript, and to acknowledge the receipt of a D.S.I.R. Research Studentship during the tenure of which most of this work was carried out.

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The Genetic Control of Conidiation in a Heterokaryon of *Neurospora crassa*

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SUMMARY: A microconidial non-colonial heterokaryon of *Neurospora crassa* was produced from a macroconidial colonial and a macroconidial non-colonial homokaryon. The macroconidial colonial strain was found to have the genetic constitution *microconidial* (*m*) (the *pe^m* of Barratt & Garnjobst, 1949) *suppressor of microconidial* (*su^m*); *col-1* and the other *m⁺ su^{m+}; col-1⁺*. The dominance relationships of *m*, *su^m* and *col-1* with their wild type alleles and the particular nuclear ratio in which they are present determine the microconidial non-colonial phenotype of this heterokaryon. As a further consequence of the dominance relationships between *col-1* and its wild-type allele, only one of the two pleiotropic effects of the mutant gene is expressed in the heterokaryon. The enormous discrepancy between the estimates of the nuclear ratio by two different plating methods is shown to be due to an almost complete suppression of one type of homokaryotic colony by the other.

Neurospora crassa produces two types of vegetative spores, macroconidia and microconidia. These differ in size, in the number of nuclei which each contains and in the time after inoculation when they first appear (Barrett & Garnjobst, 1949). Although the environment in which they are grown can influence the time of formation of both conidial types and the size and number of nuclei of macroconidia, the differences are sufficiently great to distinguish one from the other without ambiguity. Microconidia have only one nucleus; in macroconidia the number may vary from a mean of two when the parent culture is grown on a minimal medium to six when grown on certain 'complete' media (Huebschman, 1953).

A wild-type strain produces macroconidia almost exclusively and less than 1 % of microconidia. In order to have a culture produce predominantly microconidia another gene, which suppresses macroconidiation, such as *colonial-1* or *fluffy* must be present with the microconidial gene *m* (Barratt & Garnjobst, 1949). *Col-1*; *m* and *fl*; *m* strains produce copious microconidia together with a small and somewhat variable proportion (c. 1 %) of macroconidia, the former strain after incubation for 7-9 days (Barratt & Garnjobst, 1949).

In the course of plating experiments with mixtures of histidine-dependent and histidine-independent conidia, a number of spreading prototrophic colonies appeared which were unlike either of the two strains plated. The histidine-dependent strain (*K 26.9*) had the markers albino (*al-2*), histidineless (*K 26*), was macroconidial and had a wild-type growth rate; the prototroph (*P 37*) was non-albino, colonial (*col-1*) and macroconidial. The spreading colonies proved to be microconidial and to have a growth rate on minimal medium slightly less than that of the macroconidial histidineless strain *K 26.9* on

histidine-supplemented medium, but very much greater than that of the macroconidial colonial strain (*P37*) on minimal medium. The microconidial character of the spreading, presumably heterokaryotic, colonies was unexpected and it was therefore studied.

METHODS

All conidia before being plated were washed twice by centrifugation and re-suspension in 10 ml. quantities of sterile distilled water. Dilutions were made in McCartney bottles with appropriate agitation between dilutions to disperse clumps and to distribute conidia at random in the suspending medium. The media used were of the following composition: minimal medium (Westergaard & Mitchell's (1947) medium at pH 5.6); conidiating complete medium (Horowitz, 1947). Crosses were performed on Westergaard & Mitchell's medium at pH 6.5.

RESULTS

The spreading colonies were proved to be heterokaryons by the following observations.

(1) Microconidia formed by the spreading colonies when plated on to appropriate media gave rise to two colony types which were identical with the two strains plated. Both types were macroconidial.

(2) Cultures with all the properties of the microconidial prototrophic spreading colonies were obtained when heterokaryons between *P37* and various macroconidial histidine-dependent strains were synthesized on conidiating complete medium. The histidine-dependent strains would not grow in this complete medium, despite its histidine content, presumably because of the presence of inhibitory concentrations of those amino acids which prevent histidine uptake (Haas, Mitchell, Ames & Mitchell, 1952; Mathieson & Catchside, 1955).

(3) Patches of mycelium exhibiting the characteristics of one of the component homokaryons appeared, particularly on aged cultures. Heterokaryons often 'break down' in this way. The variant sector was always macroconidial and had the appearance of *P37* or *K26.9* or more frequently some mutant form of these. For example, sometimes a prototrophic albino wild growth was observed. This might have arisen by mutation at the *histidineless* locus in *K26.9* and subsequent selection in the unbalanced heterokaryon. On other occasions patches of macroconidial pink 'clumpy' mycelia characteristic of *m; col-1⁺; al-2⁺* (Barratt & Garnjobst, 1949) were observed. These presumably were derived from a mutation of the *col-1* in *P37* to *col-1⁺* followed by selection for the non-colonial prototrophic nuclei. These two types of variant growth in the heterokaryon, due to unbalance brought about by mutation in one of the component strains, occurred suddenly and discretely. Another type of change resulting in increased production of macroconidia by the heterokaryon occurred gradually, presumably as a consequence of a selection for the *P37* nuclei in the heterokaryon. After a number of transfers the growth habit of some stocks of the heterokaryon became progressively more colonial and

similar to *P37* and they produced fewer micro- and more macroconidia. The appearance of the heterokaryon is compared with that of the component strains *P37* and *K26.9* in Table 1.

Table 1. Comparison of the heterokaryon with its component homokaryons

<i>Neurospora crassa</i> culture	Character			
	Growth habit	Aerial hyphae	Conidia	Colour
<i>P37</i>	Colonial	Short, covering whole surface of colony	Macroconidia	Pink
Heterokaryon	Spreading	None	Microconidia	Pink-brown because of masses of microconidia covering surface of culture
<i>K26.9</i>	Spreading	Long, present mainly at aerial end of tube slope	Macroconidia	White

Genotypes of the two components of the heterokaryon

The component strains of the heterokaryon (*P37* and *K26.9*) were each examined to see whether they were heterokaryotic for *m* or whether they contained a modifier of *m*, such that *m*; *su^m* produced macro- and not microconidia. The cross of *K26.9* with a colonial microconidial strain segregated 1:1 for *m* amongst the *col* type ascospore isolates, indicating that *K26.9* did not have the gene *m*. Neither was the gene *fl* present since no fluffy colonies were observed in the 200 ascospore isolates or 250 colonies derived from single microconidia.

When *P37* was crossed with *Emerson A*, *m* and another gene which has been designated *su^m*, showed regular segregation. The data obtained from two crosses of *P37* with two wild-type strains and of *P37* with a microconidial strain are summarized in Table 2. Only those segregants having *col-1* were

Table 2. *Neurospora crassa*; segregation of *su^m* in two crosses

Cross	<i>col-1</i> segregants			χ^2 independence of <i>su^m</i> and <i>m</i>
	<i>col-1</i> ; <i>m</i> ; <i>su^m</i>	<i>col-1</i> ; <i>m</i> ; +	<i>col-1</i> ; +; <i>su^m</i> <i>col-1</i> ; +; +	
<i>col-1</i> ; <i>m</i> ; <i>su^m</i> × +; +; +	52	10	48	28.5
	30	8	25	12.7
	82	18	73	41.2
Deviation $\chi^2=41.0$, $P<0.01$; heterogeneity $\chi^2=0.2$, $P=0.65$				
<i>col-1</i> ; <i>m</i> ; <i>su^m</i> × +; <i>m</i> ; +	31	30	—	0.016

scored since microconidia are produced only in strains having the constitution *col-1*; *m*. It was not possible to differentiate phenotypically between the classes *col-1*; *m*⁺; *su^m* and *col-1*; *m*⁺; *su^m*⁺, each of which was aconidiate, and they were therefore grouped together. The data obtained from the cross of *col-1*; *m*; *su^m* and +; *m*; + show equality in the *col-1*; *m*; *su^m* and *col-1*;

m ; su^{m+} classes which would be expected if su^m segregated as a single gene independently of $col-1$, and if the classes $col-1$; m ; su^m and $col-1$; m ; su^{m+} had an equal viability. The data from the coupling crosses suggest that m and su^m are linked with a recombination percentage $p = 18.0 \pm 3.8\%$. Because of poor fertility which has developed in the $col-1$; m ; su^m stocks, repulsion data are not yet available.

Dominance relations of the genes h (K 26), $col-1$, m , su^m , and $al-2$

The K 26.9 and P 37 strains have the genotypes h ; $col-1^+$; m^+ su^{m+} ; $al-2$ and h^+ ; $col-1$; m su^m ; $al-2^+$, respectively. The phenotypic effects of each of the mutant genes are summarized in Table 3. At the particular ratio of K 26.9 to P 37 nuclei obtaining in the heterokaryon, h was 'recessive' to h^+ , su^m was 'recessive' to su^{m+} , $al-2$ was 'recessive' to $al-2^+$ but m was 'dominant' to m^+ . The two pleiotropic effects of $col-1$, namely, (a) the suppression of macroconidia, and the production of conidia in association with m , and (b) the restriction of growth to a colonial habit, showed different dominance relations. In the first mutant character was 'dominant', but in the second the mutant character was 'recessive'.

Table 3. *Phenotypic effects of h , $col-1$, m , su^m and $al-2$*

Gene	Phenotype
h	Requires histidine for growth. Growth in presence of histidine competitively antagonized by several different amino-acids (Mathieson & Catcheside, 1955).
$col-1$	Restricts growth habit and conidial production (see also m).
m	In the presence of the aconidiate genes $col-1$ or fluffy (fl) causes microconidiation. In the absence of $col-1^+$ and fl^+ has morphological effect 'clumpy' (Barratt & Garnjobst, 1949).
su^m	Interacts with m in the presence of $col-1$ to cause macroconidiation.
$al-2$	Albino.

Nuclear ratio of the heterokaryon

The nuclear ratio of *Neurospora* heterokaryons may be determined by plating either macro- or micro-conidia on suitable media and scoring the resulting colonies (Prout, Huebschman, Levene & Ryan, 1953; Sansome, 1947), or by crossing the heterokaryon with another strain and determining the proportion of perithecia segregating for either one or the other of the nuclear types. In the first mentioned method the assumption is made that the nuclei are segregated into conidia at random. This assumption can only be tested experimentally when macroconidia are used (Prout, *et al.* 1953). A further assumption that the relative proportions of the different colony types observed reflect the relative proportions of the conidia plated can also be tested experimentally. In the second method there is the basic assumption, difficult to test experimentally, that there are no differences in fertilizing ability between component nuclei of the heterokaryon. Furthermore, this method is considerably more arduous than the conidial plating one, and was not used in our investigations.

Since the heterokaryon being described produced uninucleate microconidia almost exclusively (only 1 % macroconidia), an attempt was made to determine the nuclear ratio by plating suitable dilutions of the conidial suspension on histidine-supplemented medium (30 mg. histidine/l.). Of 6500 colonies on 200 Petri plates tested, only two were albino *col-1*⁺; one was histidine dependent and the other was histidine independent. Assuming that the latter arose by back mutation from a histidine-dependent nucleus, the nuclear ratio (*h*⁺:*h*) would appear to be 1:3250.

An independent estimate of the nuclear ratio was obtained by determining the number of histidineless nuclei in the small proportion of macroconidia produced. Any macroconidium containing one of the rare histidineless nuclei could easily be recognized, since it would form a microconidial heterokaryon with a spreading growth habit. The total number of viable macroconidia in the mixture of conidia plated could be determined since macroconidia germinate more quickly than microconidia and form macroscopic colonies on conidiating complete medium 18 hr. before the latter. The nuclear ratio of five stocks of the heterokaryon, each of which grew from a single conidium, was determined and was found to be 0.024, 0.027, 0.028, 0.045, 0.045, respectively. This proportion of approximately one histidineless nucleus in 30 is very different from the estimate of 1 in 3250 from the microconidial plating experiment. The discrepancy suggested that the assumptions inherent in one of the methods were invalid. Either a high proportion of the homokaryotic *P 37* macroconidia did not form macroscopic colonies, or most of the histidineless microconidia were suppressed on the plate. The first alternative must be rejected since the number of colonies scored approximated to the total number of macroconidia plated (obtained from a direct haemocytometer count). This leaves the other alternative that the development of histidineless colonies was prevented, presumably by the few prototrophic cells present. This possibility was tested by plating mixtures of prototrophic and histidineless conidia and observing the proportions of each type which formed viable colonies.

Reconstruction experiment

Although there was reason to believe that only one in thirty of the microconidia produced by the heterokaryon was histidineless, for technical reasons, mixtures of prototrophic non-albino and histidineless albino microconidia were mixed in the proportions of 6:1 and plated on medium containing 30 mg. histidine/l. (this concentration allows *K 26* to grow at its maximum rate and was used in the heterokaryon plating experiment). The number of histidineless colonies which appeared was compared with the number on medium supplemented with a large excess of histidine (100 mg./l.). The data are summarized in Table 4.

Double the number of histidine dependent colonies appeared on the medium containing 100 mg. histidine/l. as on the medium containing 30 mg./l. The difference was highly significant, $t_{32}=6.312$, $P<0.01$. Thus it would seem that the presence of *h*⁺ colonies on the plate influences the growth of histidineless

Table 4. *Effect of the concentration of histidine in the medium on the suppression of growth of al-2; h conidia by +; + conidia*

Histidine concentration:	30 mg./l.		100 mg./l.	
	<i>al-2; h</i>	<i>+; +</i>	<i>al-2; h</i>	<i>+; +</i>
Colony type:				
Total number of colonies:	119	1297	280	1785
Mean number of colonies per plate:	7.934 ± 0.597	92.640 ± 2.059	14.737 ± 0.898	93.956 ± 2.230

Difference between the number of *al-2; h* colonies on medium containing 30 and 100 mg/l. of histidine = 6.804 ± 1.078 ; $t_{33} = 6.312$, $P < 0.01$.

colonies present so that at levels of histidine normally sufficient for optimum growth of the histidineless strain the growth of histidineless conidia is prevented.

DISCUSSION

Forced heterokaryons may be synthesized between two auxotrophic strains which differ in two biochemical genes (Beadle & Coonradt, 1944). Similarly, such heterokaryons may be formed between a prototrophic and an auxotrophic strain if the growth of the prototroph is restricted by the presence of a colonial gene such as *col-1* and the auxotroph carries the wild-type allele of the colonial gene. The heterokaryon discussed here was of this latter type.

The colonial component *P37* though macroconidial carried the microconidial gene (*m*). It was prevented from producing exclusively microconidia by the presence of the gene *su^m* which acted in a complementary fashion with *m* to induce macroconidiation. As a consequence of the differing dominance relations of *m* and *su^m* and of the pleiotropic effects of *col-1*, the heterokaryon between two macroconidial strains *P37* and *K26.9* was microconidial and had a spreading growth habit. At ratios of *col-1⁺; m⁺ su^{m+}* nuclei to *col-1; m su^m* nuclei of 0.045 and less, *m* was dominant to *m⁺* but *su^m* was recessive to *su^{m+}*. The microconidial phenotype of *col-1; m* was dominant to *col-1; m* but the growth habit effect of *col-1* was recessive to *col-1⁺*. The differing dominance relations of *m* compared with *su^m* and of the pleiotropic characters of the *col-1* gene suggest that the genes are not equally effective in producing their phenotypic effect. Segregation of the pleiotropic effects of *col-1* has not been observed in the course of a large number of crosses involving *col-1* and the fact that mutation affects all the pleiotropic characters simultaneously makes it unlikely that these several characters are due to closely linked genes.

The gene *su^m* is akin to a suppressor gene since it changes the microconidial character of the strain to the wild-type macroconidial condition. Because *m su^m* does not have the same phenotype as *m⁺*, a *col-1; m⁺* culture being aconidiate while a *col-1; m su^m* culture is macroconidial, it is perhaps preferable to refer to it as a modifier rather than as a suppressor. It is of little use as a marker because it can be scored only when *col-1* and *m* are also present.

The validity of using the terms dominance and recessiveness in the genic sense in the heterokaryons between haploid nuclei may be queried. The terms

were originally applied to intranuclear phenomena in diploid organisms but in heterokaryons they refer to internuclear effects. Moreover, in normal diploids, heterozygous for a particular gene, there is usually a constant 1:1 ratio of the two alleles, whereas in heterokaryons the nuclear ratio is usually not 1:1. Different heterokaryons involving the same components show considerable differences in this respect. Thus when specifying 'dominance' or 'recessiveness' in heterokaryons the nuclear ratio must also be specified. As might be expected 'dominance' varies with the nuclear ratio, thus as the proportion of *col-1*: *m su^m* nuclei in the heterokaryon increased the growth rate decreased and the production of macroconidia increased at the expense of the production of microconidia. This is in agreement with the observations of Barratt & Garnjobst (1949), who found the growth rate of heterokaryons having nuclear ratios greater than 1 *col-1*⁺ nucleus: 1 *col-1* nucleus was equal to that of the *col-1*⁺ strain but as the proportion of *col-1* nuclei increased the growth rate decreased. The growth rate of the heterokaryon at a ratio of 1 *col-1*⁺: 100 *col-1* was still 50 times that of the *col-1* strain though only 0.18 times that of the *col-1*⁺ strain.

The determination by plating methods of the proportion of a particular cell type in a mixture is fraught with difficulties due to cell interactions of various sorts. Cell competition between non-growing auxotrophs and prototrophs plated on minimal medium has been described by Grigg (1952). The complex interactions between diverse types of cells on the same plate has been discussed by Harper (1950). In the present instance an attempt to determine the nuclear ratio of a microconidial heterokaryon by plating the conidia on to a histidine-supplemented medium and scoring the resulting colonies failed because the prototrophic cultures interfered in some way with the growth of the histidineless ones. Reconstruction experiments, in which growth of histidineless colonies from a mixture of prototrophic and histidineless conidia plated under similar conditions was studied, verified the existence of the suppression. For technical reasons a higher proportion of histidineless conidia was used in the reconstruction experiments than would be present in the mixed conidia from the heterokaryon. It might be expected that as the proportion of histidine-dependent conidia decreased the extent of the suppression by prototrophic conidia would increase. This would explain the almost complete suppression of the histidine-dependent conidia when in the presence of thirty times their number of prototrophs as compared with partial suppression when the proportion was 1:6. A clue to the mechanism of the suppression is given by the work of Mathieson & Catcheside (1955) who found that prototrophs as well as histidineless cultures took up histidine from the medium against a considerable concentration gradient. If the prototrophic microconidia from the heterokaryon germinated sooner than the histidineless ones, and we have data which suggest this may be so, they might remove sufficient histidine from the low-histidine medium to prevent the normal growth of the histidine-dependent conidia. When the germination rates of conidia from the prototrophic and histidineless component strains of the heterokaryon were compared on solid medium supplemented with histidine (30 and 100 mg./l.) the prototrophs were found to germinate significantly more rapidly than the *K26* conidia.

When a large excess of histidine was present in the medium, presumably sufficient was left to enable the more slowly-germinating histidineless conidia to grow to macroscopic colony size. Other instances of colony interactions influencing the determination of nuclear ratios of heterokaryons have been observed (Jinks, 1952), and it is a factor which should be tested when conidial plating methods are employed for estimating nuclear ratios of heterokaryons, otherwise estimates of the proportion of a particular cell type in a population may be completely erroneous.

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Biological and Physical Properties of the Ryan Strain of Filamentous Influenza Virus

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SUMMARY: An influenza A strain, Ryan, isolated in Melbourne in 1954 contains on a particle basis about 20 % filaments. By using the electron microscope for particle counting, the efficiency as haemagglutinin of Ryan 'long' filaments (particles in which length:width ≥ 6) was estimated to be about 6 times greater than that of spheres of PR 8 virus. Allantoic fluid preparations of Ryan virus have high values for the ratio (log) EID₅₀:AD and estimates indicate that a maximum of 6 'long' filaments are equivalent to one EID₅₀, compared with 18 particles in the case of PR 8 virus or 14 particles in the case of a mutant strain of Ryan virus which exists almost exclusively as spheres. Compared with virus PR 8 spheres (16 particles:AD), spherical particles present in filamentous Ryan preparations have a low efficiency as haemagglutinin (29 to 38 particles:AD). These particles are formed before the 'long' filaments, and in fluids harvested 21 hr. after inoculation when the proportion of 'long' filaments is very low, about 9 spherical particles constitute 1 EID₅₀. For the purpose of subsequent chemical examinations filaments were isolated and concentrated by a process involving specific adsorption to and elution from red cells, followed by 3 cycles of differential centrifugation. The ratio of filaments: spheres in such preparations is about 50:50. 'Purified' filaments weigh about 30 times as much as the spherical particles present and account for over 95 % of the total weight present in purified preparations.

It is well known that recently isolated influenza A strains contain a high proportion of filamentous forms. These filaments have been shown to possess many of the biological properties characteristic of spherical influenza virus particles, such as serological, haemagglutinating and enzymic behaviour (Chu, Dawson & Elford, 1949; Bang & Isaacs, 1957; Burnet & Lind, 1957), but the question of the infectivity of filaments relative to that of spherical particles has remained in doubt. Morgan, Rose & Moore (1956) found in electron micrographs of sections of chorioallantois infected with the filamentous strain A/Persian Gulf/2/52 that while spherical particles possessed an electron opaque core, filaments appeared as empty shells. Their interpretation of this finding was that the spherical form of the virus was the elementary infectious unit and that the filamentous form was largely or completely non-infectious. Using quantitative counting techniques, Donald & Isaacs (1954*b*) calculated that about 10 filaments of the A/Persian Gulf/2/52 strain were needed to induce infection. This compared with a figure of 10 for the spheres from a good preparation of a non-filamentous strain. It is now evident that filaments readily break down during manipulation (Valentine & Isaacs, 1957; Burnet & Lind, 1957), and this indicates that the actual counts of Donald & Isaacs were over-estimates of the number of filaments originally present. Hence it appears that

less than 10 filaments of this strain are necessary to induce infection (Valentine & Isaacs, 1957).

It was desirable to have some information about the chemical composition of filaments, particularly for comparison with the properties of spherical particles. The A strain Ryan (Ryan F.), which was isolated during the 1954 epidemic in Melbourne, was studied because of the development (by passage at low dilution) of a mutant strain (Ryan Sph.) which exists almost exclusively as spheres (Burnet & Lind, 1957). The latter seemed an ideal control in both biological and chemical work for the spherical particles present in preparations of Ryan F. virus. Furthermore, preparations of Ryan F. virus had values for the ratio 50 % egg infectivity dose : agglutinating dose (EID₅₀:AD) which were as high as those given by Ryan Sph. or PR8 viruses. Preparations of the other well studied filamentous strain, A/Persian Gulf/2/52 (Persia F.), have given lower values for this ratio (Donald & Isaacs, 1954*b*).

The value of the ratio filaments : spheres in Ryan F. is 1:4 so that before chemical studies it was necessary to isolate and concentrate the filamentous particles. To put the chemical results on a quantitative basis, counting techniques were used to determine the number of particles/mg. dry weight in purified preparations. This led to the findings: (1) that the 'spherical particles' present in preparations of both crude and purified Ryan F. virus are less efficient as haemagglutinin than the spheres present in preparations of PR8 or Ryan Sph. viruses; (2) that fewer Ryan filaments than PR8 or Ryan spheres are necessary to induce infection in the embryonated egg. Details of these findings are presented in this paper; the results of nucleic acid analyses of Ryan filaments are given in the following paper.

METHODS

Strains of influenza virus. The following virus strains were used:

PR8 (classical influenza A virus strain, isolated by Francis in 1934).

Lee (classical influenza B virus strain, isolated by Francis in 1940).

Ryan (influenza A prime virus strain isolated during an epidemic in Melbourne in 1954). Two different lines of Ryan virus were used: one highly filamentous (Ryan F.), as was the virus on initial isolation; the other consisted mainly of spheres. This spherical form of Ryan virus (Ryan Sph.) gradually emerged during continued passage of the virus at low dilution, and it appears to differ from the parent Ryan only in having a much lower proportion of filaments (Burnet & Lind, 1957). The inoculum for batches of Ryan Sph. virus was a limit dilution fluid obtained after 16 successive passages at a 10^{-2} dilution, and that for Ryan F. virus was a fluid from either the fifth or sixth limit dilution passage.

A/Persian Gulf/2/52 (Persia F.). Received from Dr A. Isaacs, and passaged three times in the allantoic cavity.

Growth of viruses. The inocula for PR8 and Lee viruses were limit dilution fluids which had been kept at *c.* -70° . A fresh ampoule was thawed for each inoculation, the fluid diluted to 10^{-6} in 10 % horse-serum saline containing

antibiotics, and inoculated into the allantoic cavity of 200–300 11-day fertile hen eggs. After 2 days incubation at 35° the fluids of each batch were harvested and pooled, and immediately titrated for haemagglutinin and for infectivity in eggs.

The fluids used for the inocula of both forms of Ryan virus and for Persia F. virus were stored in glycerol at –10°. Batches of 100–200 10- or 11-day fertile hen eggs were inoculated into the allantoic cavity with a dilution of the inoculum varying from 10^{-2} to 10^{-4} , and the fluids harvested and pooled after 3 days incubation at 35°. Incubation was extended to 3 days to allow the development of long filaments (Donald & O'Dea, unpublished; quoted by Burnet & Lind, 1957). Titrations for haemagglutinin and for egg infectivity were carried out with the freshly-harvested fluid.

Preparation of Ryan antiserum. Antiserum was prepared in a rabbit against the filamentous Ryan virus. The rabbit was given two intravenous inoculations of 1 ml. each of concentrated virus, with an interval of 2 weeks between inoculations. The serum used was taken 4 weeks after the first inoculation. One part of serum was treated with two parts of crude receptor-destroying enzyme (Burnet, McCrea & Stone, 1946) for 1 hr. at 37° in order to destroy any non-specific inhibitor of haemagglutination. To ensure the specificity of the serum for Ryan virus, purified LEE virus (1×10^5 agglutinating doses/2.5 ml. undiluted serum) was added and the mixture kept at 4° for 1 hr. Free and combined Lee virus was removed by centrifugation (35,000 g, 30 min.), sodium citrate was added to the supernatant serum to give a final concentration of 2% (w/v) and the serum was heated for 30 min. at 65° to destroy the receptor-destroying enzyme. This method of preparing a strain-specific antiserum is a modification of the procedure originally used by Hirst (1952).

Physiological saline. 0.85 g. NaCl/100 ml. distilled water.

Phosphate buffered saline (pH 6.7). See Donald & Isaacs (1954a).

Bicarbonate saline. Physiological saline containing 5 mg. sodium bicarbonate/100 ml. saline; pH value c. 7.5.

Haemagglutinin titration. Serial twofold dilutions of virus were prepared in 0.25 ml. volumes in physiological saline, and to each dilution was added 0.25 ml. of a 1% suspension of fowl red blood cells. The cells were allowed to settle at room temperature, and the end-point read as the usual degree of partial agglutination. The reciprocal of the end-point dilution of the virus is the haemagglutinin (HA) titre, i.e. one agglutinating dose (1 AD). Most results given in the experimental section are the average of duplicate titrations.

Infectivity titrations. Serial tenfold dilutions of virus fluids were prepared in 10% horse-serum saline containing penicillin and streptomycin, and 0.05 ml. of each dilution was inoculated into the allantoic cavity of each of ten 11-day fertile hen eggs. After 3 days incubation at 35° the eggs were tested for the presence or absence of haemagglutinin in the allantoic fluids. The titre (EID₅₀) is expressed as the index of the reciprocal of the dilution yielding 50% positive fluids.

EID₅₀:AD ratio. Each virus fluid is characterized by its (\log_{10})

EID 50:AD ratio, i.e. (log) 50 % egg infectivity dose/ml.: (log) agglutinating dose/ml.

Centrifugation. Centrifugation was carried out in two types of centrifuges: (a) fitted with swinging buckets, mean radius = 10 cm.; (b) a Spinco model L, fitted with a no. 30 rotor, mean radius = 7.8 cm., angle of tube from the vertical axis = 26°.

Electron microscopy. A Siemen's microscope, type UM 100, was used with an accelerating voltage of 60 kV. and a 50 μ objective aperture. Pictures were taken at a final screen magnification of about 6000. All specimens to be examined were adsorbed to the stromata of fowl red blood cells prepared according to Donald & Isaacs (1954a) except that it was found unnecessary to treat the stromata with periodate. In early experiments involving large batches of allantoic fluid, samples were taken for the purpose of obtaining approximate figures for the relative proportions of filaments to spheres. The virus was adsorbed to stromata, the coated stromata placed on grids and shadowed with gold manganin. Later when it became necessary to know the actual number of particles/agglutinating dose (AD) for different preparations of virus, the following procedure was adopted. 400-800 AD of virus contained in 1 ml. fluid were added to 1 ml. of a suspension containing about 1×10^8 stromata/ml. After standing at 4° for 1-2 hr., the stromata were sedimented by light centrifugation, resuspended in phosphate buffered saline and fixed with 0.1 % (w/v) osmic acid. Titration of the supernatant fluid indicated that at least 90 % of the virus had adsorbed to the stromata. After a minimum of 30 min. fixing, the stromata were washed several times with distilled water and mounted from very dilute suspensions on collodion-coated grids. After shadowing with gold manganin, the number of virus particles/stroma was determined by counting particles casting a shadow, doubling this figure to allow for particles on the underside of the membrane and allowing for particles overlaying the nucleus, as described by Donald & Isaacs (1954a). The values thus obtained for PR8 virus were about double the figures quoted by Donald & Isaacs (1954a). With preparations of filaments it was sometimes noticed that where a filament extended beyond the red cell membrane edge, the latter gave the impression of overlaying the filament. In such cases, the unadsorbed portion of the filament was narrower and appeared to throw a longer shadow than the adsorbed portion. It appeared that either the stroma is so thin that it closely follows the contours of the virus particle or that the latter may in some cases protrude through the membrane. Owing to the wide variation in size of influenza virus particles, it was not possible to decide by the length of the shadow cast whether a particle was above or below the membrane. This was confirmed in later experiments when it was found that micrographs of a virus-coated stroma, taken before and after shadowing, showed the same number and distribution of particles (see also Tyrrell & Valentine, 1957). Subsequently, all counts were made on micrographs obtained by direct transmission. The area of stroma counted relative to the area of the whole stroma was estimated planimetrically and a further 5 % added to allow for the mound effect over the nucleus. The values quoted in Tables 1, 2 and 6 were obtained with this

technique. Plate 1, fig. 1, is an unshadowed micrograph of Ryan Sph. virus.

However, the two techniques were found to give different results when counting particles from filamentous virus preparations. Structures which were counted as rods in shadowed micrographs often appeared to be linear aggregates of spherical particles in unshadowed micrographs and were counted as spherical particles in the work reported in this paper. This, in part, accounts for the discrepancy between earlier estimates of the proportion of filaments (30 %) as compared with the value of 20 % (Table 4) obtained later when counts were made using unshadowed micrographs. We cannot explain the discrepancy between our estimate of about 30 % for the proportion of filaments in Persia F. virus and the value of 45 % quoted by Donald & Isaacs (1954b).

Statistical calculations. These were carried out according to Fisher (1954).

RESULTS

Definition of a filament. In conformity with the practice of other workers, particles having a value greater than 2 for the ratio length:diameter, are classified as filaments. It has been necessary, however, to subdivide this fraction into short and long filaments as neither preparations of filaments free from spherical particles, nor preparations of spherical particles free from short filaments, have been obtained. Supernatant fluids from centrifugation of Ryan F. fluids contain some filaments, the maximum size of the particles being about $L:D=5$. In this paper, these and shorter rods are designated as short filaments; particles of size $L:D \geq 6$ are designated long filaments. Unless otherwise mentioned, the properties found will refer mainly to long filaments.

Properties of virus in infected allantoic fluid

Examination in the electron microscope. Plate 1, fig. 1, is an electron micrograph of an allantoic fluid preparation of Ryan Sph. virus. There is a complete absence of long filaments and a small number only of short filaments (proportion = 4%; see Table 1). The picture is not unlike those given by well adapted influenza A strains.

Plate 1, fig. 2, is an electron micrograph of an allantoic fluid preparation of Ryan F. virus. The average proportion of filaments was found to be 20 % (Table 1; Fig. 2). For the reasons given in Methods, this value is lower than that previously quoted (Ada, Perry & Edney, 1957). Morphologically, Ryan filaments resembled those present in Persia F. virus. There was a scarcity of 'Archetti bodies' attached to Ryan filaments though occasionally such structures were seen detached from filaments on the red cell membrane. In direct transmission micrographs, long filaments exhibited little sign of continuous internal structure. Some filaments had a short section of greater electron density, often at one end. In some preparations, many filaments appeared to have spheres attached to the surface. In most cases, this proximity was probably accidental, but in others, the spheres seemed to be budding off from the filament.

Number of virus particles per agglutinating dose. These were estimated as described in Methods. To minimize errors, counts were done on batches of 5–10 specimens at a time, using the same preparations of fowl red cells for haemagglutinin titrations and of stromata for each specimen in a batch. Generally, PR8 virus was used as a control in all batches; in the limited number of experiments carried out, the variation between different PR8 preparations estimated on the same day was less than the variation in values given by one preparation estimated on different days. Counts on Persia F. virus were carried out for purposes of comparison with Ryan F. virus. Values for the ratio, number of particles:AD for spherical particles present in filamentous virus preparations were obtained by using the top 2–3 ml. of 6 ml. lots of virus preparations centrifuged at 10,000 *g* for 20 min. in the Spinco ultracentrifuge, leaving the brake off to allow slow deceleration. As controls, preparations of PR8 and Ryan Sph. viruses were treated similarly.

(i) *72-hr. fluids.* The result of particle counts on 42-hr. PR8 fluids and on 72-hr. fluids of Persia F., Ryan F. and Ryan Sph. viruses are presented in Table 1. The values for the ratio, number of particles:AD for PR8 and Ryan Sph. viruses agree well with the figure of 16×10^6 for PR8 virus reported by Donald & Isaacs (1954*a*). The findings that the supernatant fluids of both PR8 and Ryan Sph. have similar values indicate the homogeneity of these virus preparations. The slightly lower value given by Persia F. supernatant fluid (14×10^6) is probably due to the 8 % of short filaments present. Persia F. virus itself has a much lower value (9×10^6). In contrast to this, the figure for Ryan F. virus is similar to that given by PR8 or Ryan Sph. viruses. The value for Ryan F. supernatant fluid is, however, significantly higher than that for Ryan F. virus ($t=6$, $n=14$, $P<0.01$), PR8 virus ($t=6.3$, $n=18$, $P<0.01$) or Ryan Sph. virus ($t=5.6$, $n=10$, $P<0.01$).

Table 1. *The number of virus particles per agglutinating dose in allantoic fluid preparations of virus*

	Virus preparations							
	PR8	PR8 super-natant*	Ryan Sph.	Ryan Sph. super-natant*	Persia F.	Persia F. super-natant*	Ryan F.	Ryan F. super-natant*
Mean and s.d.† ($\times 10^{-6}$)	18 ± 4	17 ± 4	15 ± 2	17	9 ± 1	14 ± 2	14 ± 5	38 ± 10
Number of experiments	14	3	6	1	5	3	10	6
Range of values ($\times 10^{-6}$)	11–24	14–21	13–19		7–10	12–16	10–22	26–50
% filaments	<2	<2	4†	3†	27	8†	20	5†

* Supernatant refers to the top 3 ml. of 6 ml. lots of virus preparations after centrifugation at 10,000 *g* for 20 min.

† Almost entirely short filaments (see text).

$$\ddagger \text{ S.D.} = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}.$$

The efficiency as haemagglutinin of Ryan long filaments may be estimated from the data in Table 1 by assuming that both Ryan F. and Ryan F. supernatant fluids contain about 5 % short filaments so that the essential difference

between the two preparations is that Ryan F. virus contains 15 % long filaments. On this basis, we have

In Ryan F. virus 2×10^6 filaments + 12×10^6 spheres \equiv 1 A.D.

In Ryan F. supernatant fluid 38×10^6 spheres \equiv 1 A.D.

from which it is readily calculated that about 3×10^6 long filaments are equivalent to 1 A.D. A similar figure is also given by the filaments in Persia F. virus. If we take 18×10^6 as the number of spheres in a non-filamentous strain which is equivalent to 1 A.D., Ryan filaments in an allantoic fluid preparation are about six times more efficient as haemagglutinin than are such spheres. This compares with a figure of seven or eight calculated previously for Persia filaments (Isaacs, 1957; Ada *et al.* 1957).

(ii) *21- and 30-hr. fluids.* There are several possible explanations for the finding that the spherical particles present in Ryan F. preparations were less efficient as haemagglutinin than were the spheres in PR8 or Ryan Sph. preparations. One possibility which could be tested experimentally was that such a preparation is a mixture of 'normal' spheres + spherical particles which arise by breakdown of the long filaments. It was originally found by Drs Donald and O'Dea (quoted by Burnet & Lind, 1957), that after inoculation with Ryan F. virus the early yields of virus showed a very low ratio of filaments to spheres. In later harvests, this ratio increased and the filaments found became progressively longer. If the above hypothesis is correct, early fluids would mainly contain spherical particles of the usual efficiency as haemagglutinin. Eggs were therefore inoculated in the usual way and harvested at 21–23 hr. when the pools of allantoic fluids were found to have a low titre of haemagglutinin (titre at 18 hr. < 2; mean titre at 21 hr. = 25). As a control, eggs were inoculated with Ryan Sph. virus, but these were harvested at 30 hr., as fluids harvested at 21–23 hr. had haemagglutinin titres of less than 2. The results are shown in Table 2.

Table 2. *The number of spherical virus particles per agglutinating dose of Ryan virus harvested at different times after inoculation*

Virus preparation	Time of harvesting after inoculation (hr.)	Mean value and s.d. ($\times 10^{-6}$)	No. of experiments	Range ($\times 10^{-6}$)	% Filaments†
Ryan F.	21–23	29 ± 5	5	22–34	6
Ryan F. supernatant	72*	38 ± 10	6	26–50	5
Ryan Sph.	30	13	2		3
	72*	15 ± 2	6	13–19	4

* Values taken from Table 1.

† Almost entirely short filaments.

Thirty-hr. Ryan Sph. preparations have values within the range found previously for 72 hr. samples. In confirmation of the finding of Donald and O'Dea, early harvests of eggs inoculated with Ryan F. virus contain a low

proportion of long filaments. The value of the ratio, number of particles:AD for the spheres present in such preparations is lower than, but not significantly different ($t=1.8$, $n=9$, $P=0.1$) from the value of the spheres present in 72 hr. Ryan F. preparations, but it is significantly different from the value for the spheres present in Ryan Sph. virus ($t=5.2$, $n=9$, $P<0.01$) or in PR8 virus ($t=4.6$, $n=17$, $P<0.01$). Thus, spheres of low efficiency as haemagglutinin occur before the formation of long filaments. Their possible mode of formation will be discussed later. In several experiments, samples of Ryan F. harvested at 30 hr. were found to have an average haemagglutinin titre of 170 and to contain about 9 % of filaments.

Infectivity of Ryan virus particles. In contrast to the published figures for Persia F. virus (Donald & Isaacs, 1954*b*), high values for the ratio EID 50:AD characterize preparations of Ryan F. virus; it was partly for this reason that it was chosen as a suitable strain for study. In a preliminary series of experiments, a mean value of 6.0 was obtained which compares with values of 6.0 and 6.1 given by PR8 and Ryan Sph. viruses respectively (Table 3).

Table 3. *Infectivity to agglutinating dose ratios (log EID 50:AD) for PR8 and Ryan viruses*

	Virus preparations			
	PR8	Ryan Sph.	Ryan filaments (21 hr.)	Ryan filaments (72 hr.)
Mean value and S.D.	6.0 \pm 0.3	6.2, 6.2, 5.9	6.5 \pm 0.3	6.0 \pm 0.5
Number of experiments	12	3	6	9
Range of values	6.5-4.8	—	6.1-6.9	6.9-4.8

The relative infectivity of Ryan filaments could be calculated from these figures provided the infectivity of the spherical particles present in such preparations is known. It was previously assumed (Ada *et al.* 1957) that spheres present in the non-filamentary strains, PR8 and Ryan Sph. would be the best control for this purpose. It is now known that at least with respect to their efficiency as haemagglutinin, the spherical particles in Ryan F. preparations differ from those in PR8 and Ryan Sph. viruses. Consequently infectivity determinations were carried out on a number of 72 hr. Ryan F. fluids and on the supernatant fluids derived from them by centrifugation (see previous section). The titrations were carried out on both samples in each experiment using a common pool of eggs. Nine of the ten determinations are reported in Table 4; the tenth determination was discarded for technical reasons.

The difference of 0.21 log. units between the two mean values is significant and their values can now be used to determine the infectivity of the Ryan long filaments as follows: number of particles/AD for Ryan F. virus = 14×10^6 , for Ryan F. supernatant fluid = 38×10^6 (Table 1). EID 50:AD for Ryan F. virus = 0.6×10^6 for Ryan F. supernatant fluid = 1×10^6 (Table 4).

Table 4. *Infectivity to agglutinating dose ratios (\log_2 EID50:AD) of preparations of Ryan filaments and of the spherical particles present in such preparations.*

Virus preparations	Experimental values	Mean*	$\bar{x} - x'$ *	t^*	p^*
72 hr. Ryan F.	5.0, 5.7, 6.4, 5.3, 4.9, 6.8, 5.8, 6.2, 6.0	5.79	} 0.21	2.4	0.05
72 hr. Ryan F. supernatant	5.1, 6.2, 6.8, 5.6, 5.3, 6.4, 6.0, 6.3, 6.3	6.0			

* Fisher (1954) p. 119.

Therefore, the number of particles:EID 50 for Ryan F. virus = 23 and for Ryan F. supernatant fluid = 38.

Of this figure 23 for Ryan F. virus, 85 % or approximately 20 particles were spheres and contributed 20/38 or 0.5 EID 50. Three long filaments will then contribute 0.5 EID 50 or 6 long filaments will be equivalent to 1 EID 50. This compares with 18 PR8 particles or 14 Ryan spheres. It is interesting to note that the spherical particles present in 21 hr. Ryan F. fluid have a (\log) EID50:AD ratio of 6.5 (Table 3). Despite the fact that 29×10^6 of these particles constitute 1 AD, it may be calculated (as above) that about 9 particles are equivalent to 1 EID 50. The lower infectivity of the spherical particles in 72 hr. fluids is discussed later.

Properties of purified virus.

Purification of virus. Lee, PR8 and Ryan Sph. viruses were purified according to the procedure described previously (Ada & Perry, 1954, 1956). This involved specific adsorption to and elution from human red cells followed by two cycles of differential centrifugation (virus sedimentation; 38,000 g, 30 min; clarification of resuspended virus, 7000 g, 10 min).

In early experiments, in which Dr H. B. Donald took part, several methods designed to separate the filaments from spherical particles in Ryan F. virus were tried. These involved filtration through different materials and centrifugation in media of different densities; none was successful. In view of the size difference between spheres and filaments, separation by differential centrifugation seemed feasible. Donald & Isaacs (1954*b*) obtained a slightly higher ratio of filaments to spheres in deposits following centrifugation of infected allantoic fluids (A/Persian Gulf/2/52), but the supernatant fluids still contained appreciable number of filaments. It was thought that this poor enrichment of filaments in the deposit might be due both to the small centrifugal force used and to the very low concentration of virus in solution which, being insufficient to form a stable sedimenting boundary, would allow considerable convection to take place. In the purification scheme outlined below, 20-fold higher concentrations of virus were achieved by using a red-cell eluate of the virus and this was subjected to a considerably higher centrifugal force than that used by Donald & Isaacs (1954*b*).

Infected allantoic fluid (c. 2 l.) was passed through a fine cheese cloth chilled, and an equal volume of cold saline added. Packed human red cells

(freshly obtained) were added in the proportion of 10 ml./l. fluid with a haemagglutinin titre of 200. The container was occasionally shaken gently during a period of 2 hr. at 4° after which the agglutinated cells were recovered by brief centrifugation and washed with chilled physiological saline. The virus was eluted by suspending the cells in 60 ml. bicarbonate saline and incubating

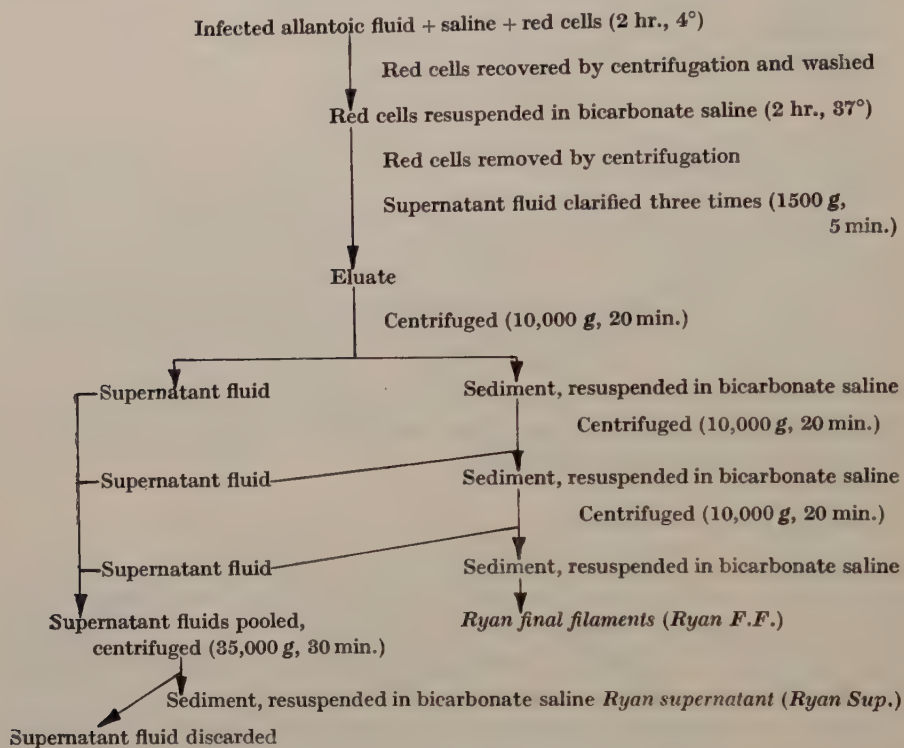


Fig. 1. Scheme for purification of filaments.

at 37° for 2–2.5 hr. The cells were sedimented in a swinging-bucket centrifuge at room temperature (1500g, 10 min.). All subsequent operations were carried out at or near 4°. The eluate was clarified by two further centrifugations as above. The solution at this stage showed marked streaming effects when disturbed.

Samples (20 ml.) of the supernatant fluid were centrifuged in the No. 30 rotor of the Spinco ultracentrifuge (10,000g, 20 min.), the brake being left off to allow slow deceleration. The supernatant fluid was placed on one side, the residue resuspended in bicarbonate saline by gentle pipetting and the volume adjusted to 20 ml. This centrifugal cycle was repeated twice more, the final sediments being resuspended in 5–10 ml. bicarbonate saline; this solution is referred to as Ryan final filaments (Ryan F.F.). The supernatant fluids from the three sedimentations were pooled and centrifuged (35,000g, 30 min.). The sediment was suspended in 5–10 ml. bicarbonate saline; this solution is referred to as Ryan supernatant (Ryan Sup.). To facilitate later reference, a flow sheet of the process is given in Fig. 1.

Yield of purified virus (recovery of haemagglutinin). Purified Ryan Sph. virus was obtained in yields of approximately 40% (see Table 5). This is comparable with results obtained earlier (Ada & Perry, 1956) with other strains of influenza A virus, the main loss being due to incomplete resuspension of virus at the clarification stages.

Table 5. *Haemagglutinin titres of infected allantoic fluids and yields of purified Ryan virus*

Virus	HA titre of allantoic fluid	Yield of purified virus (recovery of haemagglutinin)			
		Ryan spheres	Ryan filaments eluate	Ryan supernatant	Ryan final filaments
Ryan spheres	900	42 %	—	—	—
Ryan filaments	230 (75-360)	—	55 %	34 %	25 %
		59 %			

The yields of Ryan Sup. and Ryan F.F. are shown in the same table. The loss appears to occur entirely at the eluate stage. The 'recovery' of haemagglutinin from this stage may be higher than the figure in Table 5 suggests, since electron micrographs indicate that resuspension of sedimented filaments to a 'single particle suspension' is rarely achieved.

Examination of purified virus in the electron microscope. Plate 1, fig. 3, is an electron micrograph of a purified preparation of Ryan Sph. virus. The proportion of short filaments to spheres is very low. Plate 1, fig. 4, shows a preparation of Ryan Sup. There is a very small proportion of long filaments but a relatively high proportion of short filaments (about 20%; see text, Fig. 2.) A preparation of Ryan F.F. is shown in Pl. 1, fig. 5. Here the proportion of filaments to spheres is approximately 1:1. In general, the length of the long filaments is reduced, presumably due to breakdown during purification. The ratio of long to short filaments is about 2:1 (Fig. 2).

Table 6. *The number of virus particles per agglutinating dose of purified preparations of Ryan virus*

Virus preparations	Mean value and s.d. ($\times 10^{-6}$)	No. of experiments	Range ($\times 10^{-6}$)	% filaments
Ryan F.F.	10 ± 2	5	(7-14)	48
Ryan F.F. supernatant	33 ± 5	6	(25-38)	2*

* Almost entirely short filaments (see text).

Number of particles/agglutinating dose of purified virus preparations. Counts were carried out on preparations of Ryan F.F. and on preparations of the supernatant fluid derived from Ryan F.F. by centrifugation at 10,000g for 20 min.; the results are given in Table 6. Calculations from this figure indicate that the filaments present in Ryan F.F. are 5-6 times more efficient as haemagglutinin than are the spheres present in such preparations; this result is used in later calculations of the average weight of filaments in Ryan F.F. Though

this value refers to a mixture of long and short filaments, the former predominate in number so that it seems likely that this decrease in efficiency as haemagglutinin is due to a partial breakdown of the long filaments.

Breakdown of filaments during purification. As already seen the proportion of short filaments to spheres in Ryan Sup. (fractionation procedure) is greater than the proportion in Ryan F. fluids (Fig. 2); this can only arise by breakdown of filaments. It is also likely that some of the spherical particles in

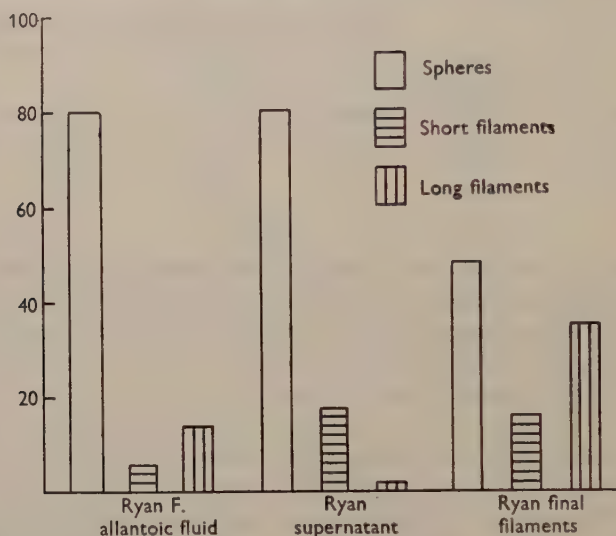


Fig. 2. Proportion of spheres, short and long filaments per 100 virus particles in crude (allantoic fluid) and purified preparations of filamentary Ryan virus.

Ryan Sup. may also have been derived from filaments. The following experiments were carried out in an attempt to see whether such a process could occur to an appreciable extent during the latter stages of purification. Essentially, the experiments tested the efficiency of the centrifugation procedure in separating spherical particles from filaments. (1) An allantoic fluid preparation of Ryan Sph. virus was purified by the method described for Ryan F. virus. The distribution of haemagglutinin in the two fractions corresponding to Ryan F.F. and Ryan Sup. was 9 and 91 % respectively. (2) An eluate of Ryan F. virus was divided into two portions, each with 19.2×10^4 A.D. contained in 40 ml. To one was added 10×10^4 A.D. of purified Lee virus contained in 0.25 ml. Both solutions were then subjected to the standard differential centrifugation procedure, the haemagglutinin titre of each fraction being determined in the presence and in the absence of a potent anti-Ryan serum. At a dilution of 1:2000 this serum decreased the haemagglutinin titres of Ryan virus by approximately 100-fold and Lee virus by two-fold (see under Methods). The haemagglutinin titres of the Ryan filaments in the presence of antiserum acted as controls to those given similarly by the Ryan and Lee mixtures, thus allowing the amount of Lee virus in the different fractions to be

calculated. Over 90 % of the Lee virus was recovered in the final fractions and showed the following distribution: Ryan Sup., 80 % Ryan F.F., 20 %. This figure of 20 % represents an upper limit due to the high sedimentation content of influenza B strains compared with that of A strains. It is clear then that between 10 and 20 % or an average of 15 % of the spheres present in the eluate may be recovered in the Ryan F.F. fraction. It is difficult to determine accurately what actual proportion of the spheres present in the Ryan F.F. fraction this would represent but it is obviously a substantial portion. It is unlikely then that an extensive breakdown of filaments into spherical particles occurs during the centrifugation procedure.

Infectivity of purified virus preparations. In several experiments, the EID50:AD ratios of Ryan F. and the corresponding Ryan F.F. preparations were determined. The values of this ratio for two preparations of Ryan Sph. virus were also determined. The results are given in Table 7. The results of the

Table 7. Comparison of the values of the log EID50:AD ratios of crude and purified preparations of Ryan virus

Virus preparation			
Ryan filaments		Ryan spheres	
Allantoic fluid	Ryan final filaments	Allantoic fluid	Purified virus
6.1	5.8	6.2	5.9
6.1	5.9	5.9	5.9
5.4	5.4	—	—
5.6	4.9	—	—
5.5	5.3	—	—
4.9	5.3	—	—
6.2	5.1	—	—
Mean values 5.7	5.4	6.05	5.9

Ryan Sph. determinations suggest that a small loss of infectivity occurs during purification. Similar values have been previously obtained with PR8 virus (Ada & Perry, unpublished). In the case of Ryan F. and Ryan F.F., the mean difference is slightly greater, suggesting that filaments in Ryan F.F. may be less infective than those in Ryan F. fluids. In three experiments, the (log) EID50:AD values of preparations of Ryan F.F. and of the corresponding Ryan F.F. supernatant fluids were determined and found to be 5.1, 5.1; 5.8, 5.8; 5.3, 6.1. Clearly, mean values calculated from these figures have little meaning and cannot be used with confidence to calculate the infectivity of the filaments in Ryan F.F.

Agglutinating doses/mg. dry weight of virus. The final fractions were acidified with trichloroacetic acid, the precipitate recovered by centrifugation, suspended in and dialysed against distilled water and dried *in vacuo* from the frozen state (Ada & Perry, 1954). Final drying was accomplished at room temperature in a vacuum desiccator over concentrated sulphuric acid. From these weights, the values of the ratio, AD:mg. dry weight, were calculated and are given in Table 8. The value for Ryan Sph. virus is only slightly lower than

that found previously for other A strains. Ryan F.F. has a relatively constant but much lower value. The value for Ryan Sup. is variable and intermediate between the other two.

Table 8. *Agglutinating doses/mg. dry weight of purified preparations of PR8 and Ryan viruses*

	PR8* ($\times 10^{-4}$)	Ryan spheres ($\times 10^{-4}$)	Ryan supernatant ($\times 10^{-4}$)	Ryan final filaments ($\times 10^{-4}$)
No. of experiments	—	3	5	11
Mean value	9	6.9	4.6	1.1
Range	6-12	5.5-8.1	2.2-7.7	0.8-1.7

* Ada & Perry, 1956.

Relative weights of spheres and filaments. Chemical analysis yields results which are often expressed in terms of weight or referred to some major constituent. In the case of preparations which are heterogeneous but may be divided into classes, it is essential to be able to express the results on a molecular or particle basis. This applies to the analysis of Ryan F.F. which for this purpose is conveniently divided into spherical particles and filaments, the latter ranging in length from 2 to about 100 times the diameter of a virus sphere.

From the number of particles/AD (18×10^6) and the number of AD/mg. dry weight (9×10^4), the average weight of one PR8 sphere is calculated to be about 6×10^{-10} μ g. This compares with a figure of 5×10^{-10} μ g. for the particle weight of PR8 virus, calculated from the light absorption data of Oster (1946). Similarly, the average weight of a sphere in Ryan Sph. is 9.5×10^{-10} μ g. while that for a particle in Ryan Sup. is 5.8×10^{-10} μ g. As Ryan Sup. contains 20 % short filaments, the average weight of a sphere in Ryan Sup. would be less than 5.8×10^{-10} μ g. From Table 8, the number of AD/mg. dry weight of Ryan F.F. is 1.1×10^4 . As this preparation is about 50 % spheres and as these filaments are about 5 times more efficient as haemagglutinin than are the spherical particles in Ryan Sup., the contribution made by spheres to 1 AD will be $\frac{1}{5}(1 \times 1.1 \times 10^4) = 0.18 \times 10^4$ AD. This corresponds to

$$0.18 \times 10^4 \times 32 \times 10^6 \times 5.8 \times 10^{-10}$$

or about 34 μ g. Therefore, 966 μ g. is due to 0.92×10^4 AD filaments. Knowing that about 6 filaments in Ryan F.F. are equivalent to 1 AD it can be readily calculated that one filament weighs about 180×10^{-10} μ g. or about $180:5.8 = 30$ times as much as one sphere (as in Ryan Sup.). Thus, filaments in Ryan F.F. account for over 95 % of the total weight present.

DISCUSSION

Many of the points of interest arising from this investigation are best considered with the results of the nucleic acid estimations presented in the following paper. There are several points, however, which may be conveniently discussed here. It was earlier estimated that between 2 and 3 Ryan filaments

were necessary to induce infection in the embryonated egg. This was based upon a mean value of 6.0 for the (log) EID₅₀:AD ratios given by 19 preparations of 72-hr. Ryan F. virus and on the calculation from the figures of Donald & Isaacs (1954*b*) that each filament was eight times more efficient as haemagglutinin than was a spherical particle in a non-filamentous strain, PR8 (Ada *et al.* 1957). In the present study, Ryan long filaments were found to be about six times more efficient as haemagglutinin than were PR8 spheres. Furthermore, nine subsequent determinations of the (log) EID₅₀:AD ratio of Ryan F. preparations gave a mean value of 5.79. The difference in the mean values of the (log) EID₅₀:AD ratios for Ryan F. and Ryan F. supernatant was counterbalanced by the fact that the spheres, of which the latter fraction was largely composed, were less efficient as haemagglutinin than were PR8 spheres. Using these figures in the calculation, an estimate of six Ryan filaments:EID₅₀ was obtained. Clearly it is not possible to settle on a definite figure but it can be confidently stated that the probability of infection in the embryonated egg by a Ryan filament is considerably greater than is the case with spherical particles present in either Ryan Sph. or PR8 preparations. The general observation (Bang & Isaacs, 1957; Valentine & Isaacs, 1957; Burnet & Lind, 1957) that filaments readily break down would of course tend to reduce this estimate of 6 particles/EID₅₀. This finding, together with the previous observation of Donald & Isaacs (1954*b*) that 10 Persia filaments were equivalent to 1 EID₅₀, tends to make untenable the suggestion of Morgan *et al.* (1956) that filaments are a non-infectious form of virus. This point will be discussed further in the following paper.

An unexpected difference between preparations of Ryan F. and Persia F. viruses is the finding that, in contrast to the spherical particles present in Persia F. fluids, those present in Ryan F. preparations (both 21- and 72-hr. fluids) were significantly less efficient as haemagglutinin than PR8 spheres. In 21-hr. fluids where there was a very low proportion of long filaments, about 9 spherical particles were equivalent to 1 EID₅₀. Thus the low efficiency as haemagglutinin does not seem to influence the infectivity of these particles. At 72 hr. this figure had decreased fourfold (see p. 29). At first sight, this could be attributed to two causes. The average haemagglutinin titre of 72-hr. Ryan F. fluids was 230 (Table 5). In such fluids, 15% of the particles are long filaments which are about 12 times more efficient as haemagglutinin than are Ryan F. spheres. Thus the spheres in these preparations contribute a haemagglutinin titre of about 75. The average haemagglutinin titre of 21 hr. fluids was 25 so that the number of spheres present at 21 hr. is increased threefold by 72 hr. If it be postulated that the later-formed particles are non-infectious, the drop in infectivity of the spheres from 21 to 72 hr. would be threefold. A complete change in infectivity of spherical particles produced before and after 21 hr. seems very unlikely. The alternative hypothesis—deterioration in chemical or physical properties—is probably largely if not completely responsible for the decrease in infectivity. The spherical particles in Ryan F. fluids may be especially susceptible to such deterioration but there is no evidence that this is the case.

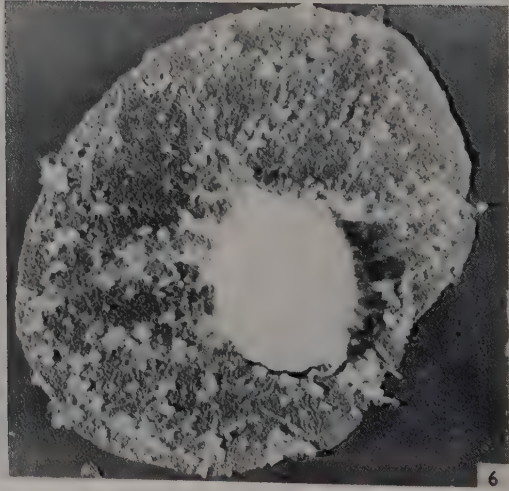
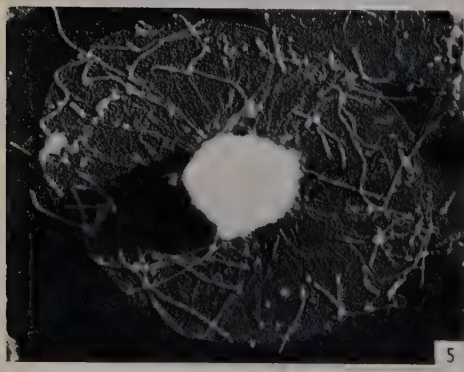
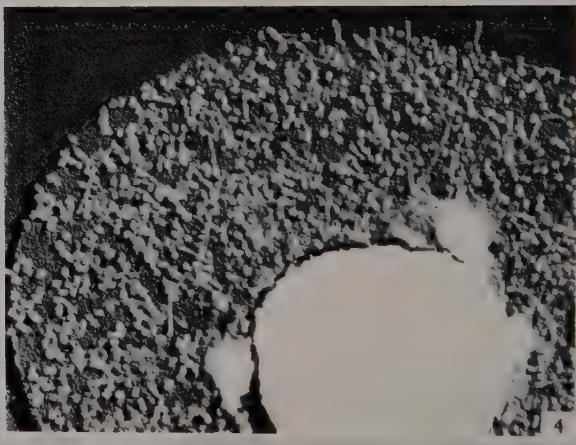
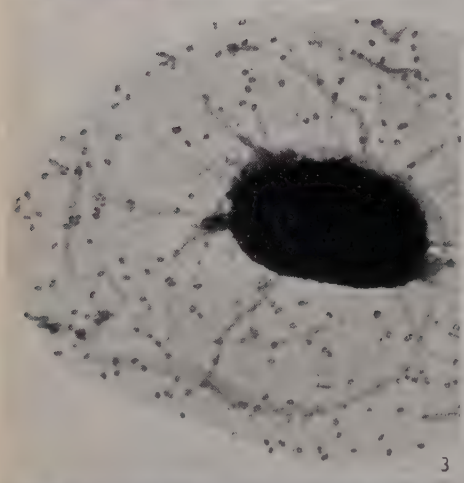
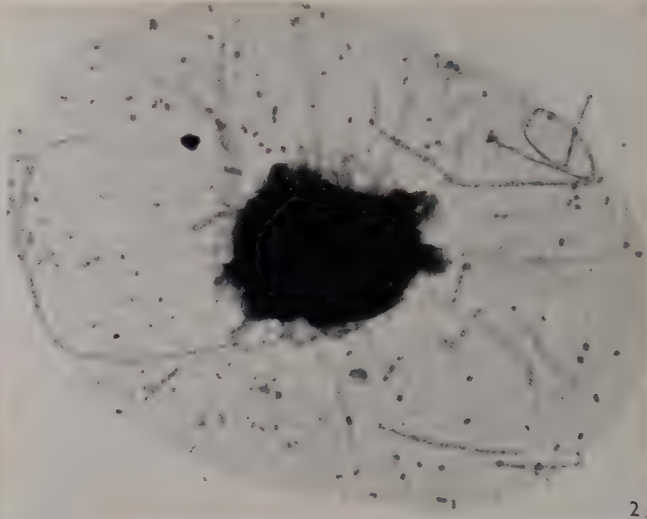
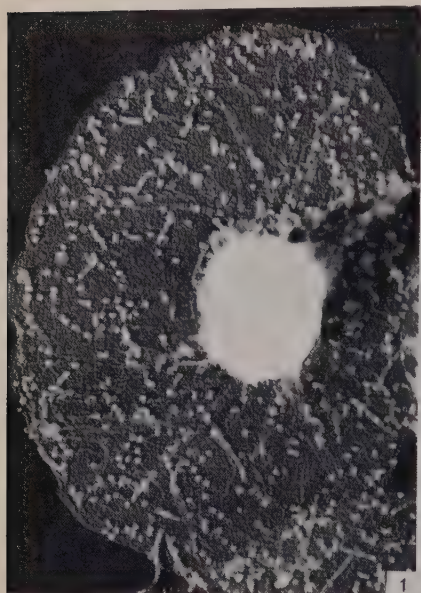
One point necessitating comment is the wide range of values for the (log) EID50:AD ratio given by 72-hr. Ryan F. preparations. The figures vary from 6.9 to 4.9 and apply both to the spherical and filamentous particles (see tables 3 and 4). Deterioration in the value of this ratio is often regarded as evidence of 'thermal denaturation' (Horsfall, 1954). Despite the fact that the bulk of the particles present in Ryan F. are produced during the first 30 hr. incubation and are then subjected to a further 30 hr. at 35°, 10 of the 29 values quoted for the (log) EID59:AD ratios were 6.2 or greater. Clearly other factors as well as temperature of incubation affect the infectivity of the virus. This wide range of values is not mainly due to variation in eggs, inconsistency of technique, etc., as 21-hr. Ryan F. fluids which were titrated at the same time as 72-hr. Ryan F. fluids, consistently had high values for the (log) EID50:AD ratio (Table 3). Obviously there has been little chance of particle deterioration in 21-hr. fluids. Thus if preparations of Ryan F. containing mainly 'undamaged' particles have values for the (log) EID50:AD ratio of 6.2 or greater, the estimate of 6 long filaments/EID50 would be greatly reduced.

These results further emphasise that quoting values for the ratio (log) EID50:AD is an incomplete description of the infectivity of a virus preparation. Particle counts are necessary to complete the picture. It has been generally considered that the average value for the (log) EID50:AD ratio of standard virus preparations is about 6.2 (see review by Isaacs, 1957). Values consistently higher than this have been occasionally obtained, e.g. with Rob virus, a B strain of recent human origin (Ledinko & Perry, 1955). The explanation for such findings may also be the presence of spherical virus particles of low efficiency as haemagglutinin.

We wish to thank Drs H. B. Donald and J. M. Edney who were concerned in the initial stages of the investigation, and Professor Sir Macfarlane Burnet, F.R.S., for his advice and encouragement. This work was aided by a grant from the National Health and Medical Research Council, Canberra, A.C.T., Australia.

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(Facing p. 39)

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EXPLANATION OF PLATE

Electron micrographs of preparations of virus adsorbed on to laked avian erythrocytes.

- Fig. 1. Ryan Spheres (72 hr.). Shadowed with gold manganin. Magnification $\times 8000$.
- Fig. 2. Ryan filaments (72 hr.). Unshadowed. Magnification, $\times 9000$.
- Fig. 3. Purified Ryan spheres. Unshadowed. Magnification, $\times 9000$.
- Fig. 4. Ryan supernatant. Shadowed with gold manganin. Magnification, $\times 12,500$.
- Fig. 5. Ryan final filaments. Shadowed with gold manganin. Magnification, $\times 6000$.
- Fig. 6. Ryan final filaments after 10 cycles of freezing and thawing (see following paper). Shadowed with gold manganin. Magnification, $\times 7000$.

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Properties of the Nucleic Acid of the Ryan Strain of Filamentous Influenza Virus

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SUMMARY: The amount, type and properties of nucleic acid have been estimated in the two end fractions, Ryan supernatant (Ryan Sup.) and Ryan final filaments (Ryan F.F.) of a purification procedure applied to allantoic fluid preparations of filamentary Ryan virus. Ryan Sup. consists of 80 % spherical particles and 20 % short filaments (length:diameter < 6). Analysis indicates an average nucleic acid content of 0.75 % (PR8 = 0.91 %). The value of the ratio, adenine + uracil: guanine + cytosine for the nucleic acid of A strains of influenza virus varies between 1.22 and 1.28. The value for Ryan Sup. is 1.20; for a mutant strain of Ryan virus which exists almost exclusively as spheres, the value is also 1.20.

The ratio of filaments to spherical particles in Ryan F.F. is about 50:50. On a dry-weight basis, Ryan F.F. contains about 0.25 % RNA. There may be small amounts of DNA present. On a particle basis, the residual filamentary structures contain about eight times as much RNA as do PR8 spheres. The value of the above ratio of bases however is about 0.9. Treatments of filaments with diethyl ether releases a soluble complement-fixing antigen (CFA) which on purification is found to have the same nucleic acid content per unit of CF activity as does soluble CFA isolated from either PR8 or Ryan Sup. viruses. In each case the value of the above ratio of bases is about 1.25. On a particle basis filaments in Ryan F.F. contain 3 to 4 times as much soluble CFA as does PR8 virus so that the residual RNA in Ryan F.F. must closely correspond in properties to RNA from the potential host cell which has a value for the above ratio of about 0.6. Exposure of Ryan F.F. to ribonuclease or to a procedure which degrades the filamentary form to smaller spherical units does not affect the amount or properties of the associated RNA.

A tentative scheme is proposed for the formation of virus particles of Ryan F. preparations. It is postulated that most of the spheres present in such preparations arise by fragmentation of the tip of forming filaments where there is a relative concentration of viral type RNA. The filaments which are found in the allantoic fluid thus represent only part of the original filamentary structures. This concept implies that filaments break more readily at those places where there is an enrichment of viral type nucleic acid.

Purified influenza virus preparations have been shown to contain carbohydrate, lipid, protein and nucleic acid. Some of these have been examined in detail, for example: amino acids (Knight, 1947), carbohydrate (Ada & Gottschalk, 1956; Frommhagen & Knight, 1956) and nucleic acid (Ada & Perry, 1954, 1956). The nucleic acid is the only component which has been shown to possess properties which clearly distinguish it from the corresponding component in the potential host cell. Spherical forms of influenza virus contain about 1 % RNA which features a characteristic proportion of nitrogenous bases—a pattern which is sharply distinct from that of the host cell RNA.

Evidence accumulated over the past few years indicates that the influenza

virus particle is assembled at or near the host cell wall and it is possible that components from the latter (e.g. lipid, mucoprotein) are incorporated into the virus membrane. The demonstration of a parallelism between the haemolytic power of various substances for chick erythrocytes and their capacity to damage influenza virus filaments suggests that the filament surface is less different from the host cell surface than is that of the spherical particles (Burnet, 1956). This concept implies that filaments contain an appreciable proportion of relatively unmodified host cell components. An estimate of the amount and localization within the filament of these and of specific virus components would throw light on the structure of a filament. Due to the large difference in their properties, spherical virus type RNA and host cell type RNA are suitable markers in such an investigation. The finding (Ada, Perry & Abbot, 1958) of the infectious nature of Ryan filaments enhanced the interest attached to a study of the amount, type and properties of nucleic acid present in purified preparations of Ryan filaments. The results of such an investigation are reported in this paper.

METHODS

Virus. The strains of virus used, the conditions of growth, methods of estimation of haemagglutinin and infectivity titres are described in the previous paper (Ada *et al.* 1958).

Virus fractions. The fractionation procedure applied to allantoic fluid preparations of Ryan F. virus was described previously. Two fractions were obtained, namely: Ryan Sup. which consisted of 80 % spheres and 20 % short filaments, and Ryan F.F. which was a 1:1 mixture of spheres and filaments (Ada *et al.* 1958).

Physiological saline. 0.85 g. NaCl/100 ml. distilled water.

Bicarbonate saline. Physiological saline containing 5 mg. sodium bicarbonate/100 ml. saline; pH value *c.* 7.5.

Calcium magnesium saline (Ca Mg saline). Prepared according to Mayer, Osler, Bier & Heidelberger (1946) as follows: NaCl, 9.0 g.; 5:5-diethyl barbituric acid 0.575 g.; Na-5:5-diethyl barbiturate, 0.375 g.; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.168 g.; CaCl_2 , 0.028 g.; made up to 1 l. with distilled water. After autoclaving, the solution had a pH value of 7.2.

Complement-fixation titration. The micro-technique used was first described by Donnelley (1951). Serial twofold dilutions of antigen were prepared in volumes of 0.04 ml. in Ca Mg saline, 0.04 ml. of complement diluted to 3 HD 50 was added to each tube, and then 0.04 ml. of 1/5 or 1/10 rabbit antiserum. The tubes were incubated for 1 hr. at 37°, and then 0.04 ml. of 3 % sensitized sheep red cells were added to each tube. The test was read after a further 30 min. incubation. The titre is expressed as the reciprocal of the antigen dilution showing 50 % haemolysis.

Nucleic acids. Purified yeast nucleic acid (YNA) and deoxyribonucleic acid (DNA) from calf thymus were purified as described previously (Ada & Perry, 1954, 1956).

Extraction and estimation of nucleic acid from virus. The nucleic acid was extracted from the dried virus using a hot 10 % (w/v) NaCl solution. The

amount present in the salt extract was determined by using the formula $Ep = 30.98 E/cl$ (Chargaff & Zamenhof, 1948), where Ep = atomic extinction coefficient at 260 $m\mu$, with respect to phosphorus, E = optical density, c = concentration of phosphorus in g./l., and l = thickness of absorbing layer. Details have been given previously (Ada & Perry, 1954, 1956).

Paper chromatography. The nucleic acid samples were hydrolysed and the purine and pyrimidine derivatives separated by paper chromatography. The separated components were located by their absorption in ultraviolet light, eluted with 0.1 N-HCl and the optical density of the eluate read at the wavelength of maximal absorption for each derivative (Ada & Perry, 1956). The precaution was taken of checking the absorption ratio of each derivative as isolated from the chromatographic paper. In order of increasing R_F values guanine was read at 248 and 262 $m\mu$, adenine at 248 and 262 $m\mu$, cytidylic acid at 262 and 278 $m\mu$ and uridylic acid at 262 and 278 $m\mu$. Low values for the ratios of the virus derivatives compared with the values given by the derivatives of a sample of YNA treated in a similar fashion, indicated either faulty separation or contamination with other u.v. absorbing substances.

In one instance a departure was made from the technique outlined above. Samples of the soluble complement-fixing antigen derived by ether treatment from Ryan Sup. and Ryan F.F. were obtained in small amounts. To avoid losses involved in precipitating the nucleic acid present in low concentration in the 10 % salt extract, the whole complex was digested with N-HCl and run on paper. As a control, an equal weight of bovine serum albumin was digested and run on paper. The absorption ratios of the isolated purine and pyrimidine derivatives, when adjusted by using the values of the protein control, agreed well with the YNA values in the case of guanine and adenine but were 12 and 17 % lower than the YNA values in the case of the cytidylic acid and uridylic acid derivatives, respectively. Estimates of the amounts of these two components were therefore too high, but this effect was largely abolished when calculating the value of the ratio adenine+uracil:guanine+cytosine, and was allowed for when determining the total amount of nucleic acid associated with the soluble complement-fixing antigen.

RESULTS

Chemical properties of purified virus

Lipid content. Prior to the determination of the nucleic acid content, the dried virus was defatted with a chloroform + methanol mixture (Ada & Perry, 1954). The difference in weight before and after such treatment gives an approximate value for the lipid content of the preparation. In two experiments, the following values were obtained: Ryan Sup. 48 and 51 %; Ryan F.F., 54 and 59 %. These values are higher than the mean value of 44 % quoted earlier for PR 8 (Ada & Perry, 1954).

Nucleic acid content. The nucleic acid content of the defatted virus preparation was determined as described in Methods. Extraction with a 10 % salt solution was found to give a less accurate estimate in the case of filament

preparations than with spherical viruses. With the latter it was shown (1) that the u.v. spectrum of the salt extract agreed well with that of a purified sample of yeast nucleic acid (YNA) and (2) that material which absorbed with a maximum at $260\text{ m}\mu$ was quantitatively extracted by the hot salt treatment. Thus, measurement of the optical density at $260\text{ m}\mu$ of the salt extract gave a reliable estimate of the nucleic acid content (Ada & Perry, 1954, 1956).

In the present investigation, similar conditions applied to preparations of Ryan spheres and, to a lesser extent, to Ryan Sup. In the case of final Ryan filaments, however, the u.v. absorption curve was much flatter ($260\text{ m}\mu/280\text{ m}\mu = 1.4\text{--}1.5$) than that of the control YNA ($260\text{ m}\mu/280\text{ m}\mu = 2.08$), indicating the presence in the extract of other u.v. absorbing substances. Secondly, extraction with hot perchloric acid (Ogur & Rosen, 1950; Martin & Morton, 1956) of the salt extracted virus residue indicated that small amounts of nucleic acid (or material with a maximum absorption at $260\text{ m}\mu$) was not extracted by salt solution. At most this amounted to 12 % of that present in the salt extract and because of the small amount involved (max. $12\text{ }\mu\text{g.}$) was not further examined. An alternative method for estimating the amount of nucleic acid present in filaments was to determine the recovery of the nitrogenous bases following chromatography of the acid-digested nucleic acid. This gives a minimum value owing to incomplete precipitation of the nucleic acid by ethanol. In the case of two preparations of Ryan Sph. virus the recovery amounted to over 90 % of that estimated to be present from the u.v. spectrum of the salt extract. Values of 60–70 % were similarly found for two preparations of Ryan F.F. In Table 4 where the values for the nucleic acid content of the virus preparations are given, a maximum and a minimum value are given for the Ryan F.F., representing the estimates from the salt extract and from the recovery of bases respectively. In later calculations, the mean of these two values is used.

The sodium chloride extracts from these batches of Ryan F.F. were tested for the presence of DNA by the method of Keck (1956). The extract from one batch gave no peak at $490\text{ m}\mu$. The extracts from the other two preparations gave definite peaks at $490\text{ m}\mu$, indicating the presence of deoxyribose. Based on these figures, the amount of DNA calculated to be present in the salt extracts of the two batches amounted to 11 and 16 % of the total nucleic acid present. These figures are considerably too high, however, as the absorption curves were found to be higher at 450 and $520\text{ m}\mu$ and yet lower at $490\text{ m}\mu$ than curves given by the appropriate control DNA solution. There may therefore be a small amount of DNA associated with filaments.

The values for the nucleic acid content of the virus preparations expressed on a percentage dry weight basis are given in Table 1. In the case of Ryan Sup. and Ryan F.F., the mean nucleic acid contents expressed on a virus particle basis are also presented, using the values for particle weights found earlier (Ada *et al.* 1958). For comparative purposes, a value is included for the nucleic acid content/PR8 particle. Though this figure is obtained from the nucleic acid content of a purified preparation of virus and the particle counts of virus present in allantoic fluid preparations, it is unlikely that any significant

Table 1. *Nucleic acid content of PR8 virus and of spherical and filamentary forms of Ryan virus*

Virus preparation	Nucleic acid content (expressed on a dry-weight basis)			Particle weight of virus* ($\times 10^{10} \mu\text{g.}$)	Mean nucleic acid content (expressed on a virus particle basis) ($\times 10^{12} \mu\text{g.}$)
	Mean (%)	No. of experiments	Range of values		
PR8†	0.91	11	0.79–1.12	6	5.5
Ryan spheres	0.87	3	0.75–1.0	—	—
Ryan supernatant	0.75	6	0.60–0.93	5.8	4.4
Ryan final filaments	$\left\{ \begin{array}{l} \text{max.} = 0.30\ddagger \\ \text{min.} = 0.20\ddagger \\ \text{mean} = 0.25\ddagger \end{array} \right\}$	8	max. 0.23–0.35	175	44

* Ada *et al.* (1958).

† Ada & Perry (1956).

‡ See text.

Table 2. *Proportion of bases in nucleic acid of PR8 and Ryan viruses*

Virus	No. of experiments	Nucleotide composition (as mole/100 mole nucleotide)				Adenine + uracil Guanine + cytosine
		Adenine	Guanine	Cytosine	Uracil	
PR8*	5	23.1 \pm 0.2	20.2 \pm 0.5	24.0 \pm 0.7	32.9 \pm 0.5	1.27 \pm 0.02
Ryan spheres	2	21.8	20.2	25.1	32.9	1.21
		22.3	20.5	24.9	32.3	1.20
Ryan supernatant	5	22.7 \pm 1.4	21.7 \pm 1.6	23.7 \pm 1.1	31.9 \pm 1.5	1.20 \pm 0.03
Ryan final filaments	6	21.2 \pm 1.3	25.3 \pm 1.4	26.2 \pm 2.1	27.3 \pm 1.5	0.94 \pm 0.05
Host cell RNA†	1	19.6	32.6	27.5	20.3	0.66

* Ada & Perry (1956).

† Ada & Perry (1955).

error is involved. Expressed on a dry-weight basis, PR8 and Ryan Sph. viruses yield comparable values while that for Ryan Sup. is only slightly less. The mean value for Ryan F.F. is less than one third as much. On a particle basis however, Ryan F.F. has a much higher nucleic acid content than Ryan Sup. Although Ryan F.F. contains about equal amounts of spherical particles and of filaments, the filaments account for over 95 % of the weight so that the nucleic acid content of a filament in Ryan F.F. will not be significantly different from $44 \times 10^{-12} \mu\text{g.}$ This figure will therefore be used in later calculations. On a particle basis the nucleic acid content of Ryan Sup. is 80 % of the value for PR8.

Proportion of bases in the virus nucleic acid. As well as yielding information concerning the identity of the bases present in the nucleic acid this analysis indicates the degree of contamination of the virus particle by the host cell type nucleic acid. Results are given in Table 2.

In every case only four spots, agreeing in R_f values with the spots from a control yeast nucleic acid digest, were present. For purposes of comparison, the values for each virus are conveniently expressed by the ratio, adenine +

uracil:guanine+cytosine. The value of this ratio given by Ryan Sph. virus and by Ryan Sup., 1.20, is close to the range previously found for A strains of virus, 1.22–1.28. Ryan F.F., however, has a proportion of bases which yields a value for the ratio which is intermediate between that of spherical viruses and the value given by the RNA of a crude extract of chorio-allantoic membrane (Ada & Perry, 1955).

Action of ribonuclease on Ryan virus. Short exposure of influenza virus (PR8) to pancreatic ribonuclease has previously been shown not to affect the nucleic acid content of the virus particle (Ada & Perry, 1954). It was of interest to see whether the exposure of filamentary forms of virus to ribonuclease affected the content or base proportions of the associated nucleic acid.

In a typical experiment, 80 ml. of an eluate of Ryan F. virus containing 200×10^4 AD was divided into two parts. To one part was added 1 ml. of saline containing 380 μ g. ribonuclease and both parts were then incubated at 37° for 2 hr. The virus in each sample was obtained by the usual procedure of differential centrifugation as described in the preceding paper (Ada *et al.*, 1958). In two cases, preparations of Ryan Sph. virus were also treated with ribonuclease, the enzyme being added at the eluate stage and the virus subsequently purified by differential centrifugation (Ada & Perry, 1956). The nucleic acid content of each virus preparation was estimated and the proportion of bases determined.

The results of several experiments are presented in Table 3.

Table 3. *Action of ribonuclease on the infectivity and nucleic acid content of Ryan virus*

Virus	No. of experiment	Amount of ribonuclease added/mg. virus (μ g.).	Length of incubation at 37° (hr.)	EID 50: AD		Nucleic acid content (%)	Ratio	Ratio
				Allantoic fluid	Purified virus		Adenine + uracil Guanine + cytosine	Purines Pyrimidines
Ryan spheres	1	15	2	6.2	6.3	0.87	1.26	0.72
	2	Nil	2½	6.2	5.9	0.87	1.20	0.74
		25	2½	6.2	5.9	0.85	1.21	0.73
Ryan final filament	1	14	1	6.8	—	0.23	0.89	0.85
	2	Nil	2½	5.5	5.4	0.30	0.99	0.80
		12.5	2½		5.3	0.33	1.03	0.83
	3	Nil	2½	6.1	5.8	0.26	0.94	0.88
		13	2½		5.1	0.27	0.94	0.86

Exposure to ribonuclease was found to leave unchanged the following properties of Ryan F.F. preparations: (1) Yield of virus and the value of the ratio, AD : dry weight of virus. (2) Amount of nucleic acid. (3) Value of the ratios, adenine+uracil:guanine+cytosine and purine:pyrimidine. As ribonuclease is unable to split the link between purine nucleotides the value of the latter ratio is a sensitive test for enzymic degradation of the nucleic acid. It can be concluded therefore that the nucleic acid has not been affected by the enzyme although the amount of the latter added corresponded in some experiments to three times the estimated amount of nucleic acid present. (4) Value of the ratio EID 50:AD. The one exception in the last experiment quoted appears to be anomalous in view of the nucleic acid results.

Degradation of filaments

Treatment with ethyl ether. It has been shown by numerous workers that under suitable conditions, exposure of influenza virus to ethyl ether results in the structural breakdown of the virus particle with a concomitant loss of infectivity and the release of a soluble haemagglutinin and a soluble complement-fixing antigen (CFA). The soluble haemagglutinin is a mucoprotein, whereas the CFA is a nucleoprotein (Frisch-Niggemeyer & Hoyle, 1956; Schafer, 1957). The latter probably contains all the nucleic acid in the virus particle and, at least in the case of PR8, the proportion of bases in the CFA nucleic acid is the same as that in the parent virus particle (Ada, 1957). It was of interest to know (1) whether filaments contain any CFA; (2) the properties of the nucleic acid (if any) associated with the CFA.

Ether treatment was carried out following a slight modification of the technique described by Schafer & Zillig (1954). Purified virus (5–10 ml.; $1-4 \times 10^6$ AD) was shaken 16 hr. at room temperature with two volumes of peroxide-free ether. After centrifugation (5000 g, 15 min.) the sedimented and interfacial precipitates were pooled, suspended in saline and this and the aqueous layer reshaken with ether as above. This procedure was again repeated. The aqueous layers obtained by centrifugation were pooled, the ether removed by brief exposure to vacuum and intact virus particles removed by centrifugation (20,000 g, 45 min.). The supernatant contained the CFA and soluble haemagglutinin. The latter was removed by exhaustive extraction with freshly washed, packed red cells. The CFA was sedimented from the extracted solution by centrifugation (98,000 g, 120 min.), resuspended in saline and tested for biological activity and the presence of nucleic acid.

Table 4. *Amount and properties of soluble complement-fixing antigen (CFA) obtained by ether treatment of PR8 and Ryan viruses*

Virus	Units of CFA released/AD of virus ($\times 10^{-3}$)	AD/mg. dry weight* ($\times 10^{-4}$)	Units of CFA released/ mg. dry weight virus	Units of CF activity/ mg. dry weight of CFA ($\times 10^{-4}$)	Nucleic acid content (%)	Ratio Adenine + uracil Guanine + cytosine
PR8	10.3, 9.1, 14.3	9	1020	1.7	4.6	1.23
Ryan supernatant	8.7	4.6	400	1.5	3.6	1.25
Ryan final filaments	12.5, 13.3, 8.6	1.1	124	0.7	2.0	1.21

* Ada *et al.* (1958).

The amount and properties of the CFA obtained in this way from PR8 and Ryan viruses is shown in Table 4. The amounts of CFA released/AD of intact virus (calculated from the CF activity of the pooled aqueous layers before removal of intact virus and haemagglutinin) are comparable in the cases studied—PR8, Ryan Sup. and Ryan F.F. When these values are converted to units of CFA /mg. dry weight virus, a big difference is immediately apparent.

The figure for PR8 is almost eight times larger than that for Ryan F.F. with Ryan Sup. in between these two. In the next column, the units of CF activity/mg. CFA are given. PR8 virus and Ryan Sup. have similar values, that for Ryan F.F. being about half as great. The values for nucleic acid content in the following column show the same trend. This has two consequences: (1) due to the fact that both the nucleic acid content and the CF activity are low, the preparation of CFA from Ryan F.F. is probably impure and not intrinsically less active than antigens from other virus preparations; (2) the close agreement between the CF activity and amount of nucleic acid associated with the antigens from the three virus preparations suggests again that the presence of nucleic acid in the antigen preparations is not fortuitous. Finally in the last column are given the values of the ratio, adenine + uracil:guanine + cytosine, which characterize the CFA nucleic acids. For reasons given in Methods, these analyses are subject to a slight error. Nevertheless, the main conclusion to be drawn is quite clear. Whereas with PR8 and Ryan Sup. antigens the values agree well with those obtained with the nucleic acid extracted from the intact virus, the value given by the Ryan F.F. antigen clearly differs from that of the intact virus nucleic acid and agrees well with that of spherical virus preparations. This not only indicates that the nucleic acid found in Ryan F.F. is heterogeneous but also that it contains a fraction with the 'viral type' proportion of bases.

It has recently been found that treatment of virus with deoxycholate also results in the release of a soluble haemagglutinin and CFA. The optimum conditions for isolation of these components have not yet been determined but the results indicated that filaments contain a soluble CFA in approximately the same proportions/AD as given in Table 4. Details of the procedure will be published later.

Freezing of filaments. Donald & Isaacs (1954) have shown that following exposure to ultrasonic vibrations, filaments break up into smaller units which are approximately spherical. This results in an increase in haemagglutinin titre, although the infectivity is not affected. It is possible that such a process might liberate any nucleic acid or nucleoprotein either adsorbed to the filaments or in a situation where, following fragmentation of the filaments, diffusion into the medium could take place.

As a suitable ultrasonic generator was not available, purified virus was subjected to numerous cycles of freezing and thawing in a bath of ethanol and solid CO₂. While the appearance of Ryan Sup. preparations was unchanged, the marked streaming shown by Ryan F.F. decreased until between the 6 and 10th cycle, it was no longer apparent. Following this treatment, the virus preparations were re-adsorbed on to red cells and after elution (1½ hr. 37°), the eluate was centrifuged (35,000g, 30 min.). The biological properties of the deposited virus were tested and the nucleic acid investigated using the procedures described earlier.

The results are given in Table 5. In columns 3*a* and 3*b* the amount of haemagglutinin was estimated from the haemagglutinin titre of the solution immediately before and after the freezing and thawing process. In both

Table 5. *The effect of repeated cycles of freezing and thawing on some biological and chemical properties of Ryan filaments*

No. of experiments	Virus	No. of cycles of freezing and thawing	Amount of haemagglutinin		Recovery of haemagglutinin after freezing and thawing	(log 10) EID 50 purified virus		AD/mg. dry weight		Nucleic acid content of virus		Ratio	
			Before ($\times 10^{-3}$)	After ($\times 10^{-3}$)		Before ($\times 10^{-4}$)	After ($\times 10^{-4}$)	Before (%)	After (%)	Adenine + uracil			
										Guanine + cytosine			
1	Ryan supernatant	6	320	280	—	—	—	—	—	—	—	Before	After
	Ryan final filaments	6	160	340	—	—	—	—	0.30† (0.23-0.35)	0.33	—	—	—
2	Ryan supernatant	10	38	22	52	9.6	9.0	—	—	—	—	—	—
	Ryan final filaments	10	19	45	150	8.8	8.6	—	—	—	—	—	—
3	Ryan supernatant	10	115	112	56	—	—	4.6 (2.2-7.7)	6.7	0.75† (0.6-0.93)	0.8	—	—
	Ryan final filaments	10	60	180	136	—	—	1.1 (0.8-1.7)	4.7	0.30† (0.23-0.35)	0.3	0.94‡	1.04

* Mean value and range from Table 8. (Ada, Perry & Abbot, 1958).

† Mean value and range from Table 1.

‡ Mean value from Table 2.

preparations of Ryan Sup., there was no increase in titre. In the case of Ryan F.F., however, the titre rose two to threefold and in the second experiment, this is reflected in the yield of haemagglutinin as estimated from the haemagglutinin titre of the final sedimented virus. As filaments in Ryan F.F. are about 6 times more efficient as haemagglutinin than are the spheres (Ada *et al.*, 1958), it is readily seen that this treatment breaks down the filaments into particles which are equivalent (as haemagglutinin) to about 15 (i.e. $2\frac{1}{2} \times 6$) Ryan F. spheres. Ryan Sup. showed a slight drop in EID₅₀ during freezing and thawing but the value for Ryan F.F. remained unchanged. Donald & Isaacs (1954) found the infectivity of filament preparations to remain unchanged after fragmentation with ultrasonic vibrations. In the second experiment, the ratio AD/mg. dry weight was determined. The value of Ryan Sup. is within the range previously found for untreated preparations but the Ryan F.F. preparation shows an increase (three to fourfold) compared with the mean value and range of untreated preparations. The nucleic acid content of both Ryan Sup. and Ryan F.F. remained unchanged following freezing and thawing indicating that there was no preferential loss of nucleic acid or protein from the fragmented filaments. The value of the ratio, adenine+uracil:guanine+cytosine was not very different from the mean value found previously for untreated preparations.

Plate 1, fig. 6, in the preceding paper (Ada *et al.* 1958) shows an electron micrograph of a preparation of Ryan F.F. after ten cycles of freezing and thawing. No filaments remained and the picture is rather similar to that found by Donald & Isaacs (1954) after exposure of filaments to ultrasonic vibrations.

In summary then, the process of freezing and thawing, though drastically changing the morphology of filaments had not appreciably changed the content or properties of the associated nucleic acid. To this extent, these results support those from the experiment with ribonuclease in suggesting that all the nucleic acid found associated with filaments is incorporated into the structure of the virus particle.

DISCUSSION

The original aim of this investigation was to study the properties, particularly the nucleic acid content, of filamentous forms of virus. In confirmation of the experiences of previous workers, it soon became clear that filaments were fragile structures which readily fragment, e.g. during purification procedures. The smaller of these breakdown products are isolated in the fraction, Ryan Sup., which contains the bulk of the spherical particles originally present in the infected allantoic fluid. While the other end fraction, Ryan F.F., which contains a high concentration of filaments (50 %), would seem to be the more important for study, the properties of both these fractions must be taken into account when attempting to define the mode of formation and structure of a Ryan filament.

On a dry weight or a particle basis, the particles present in Ryan Sup. contain about 80 % as much nucleic acid as do PR 8 spheres (Table 1). Further-

more, the proportion of bases in the nucleic acid from the particles in Ryan Sup. is the same as that found in the nucleic acid from the mutant strain, Ryan Sph. virus, that is, adenine+uracil:guanine+cytosine=1.20. This value is only slightly lower than the range found previously for A strain viruses, 1.22-1.28. This may indicate that both Ryan preparations contain host cell type RNA but, if so, the amount involved must be very small unless it is assumed that all A strains are also contaminated. Marked contamination is unlikely in view of the observed specific difference in the nucleic acid base proportions of A and B types of influenza virus (Ada & Perry, 1955). On the other hand, Ryan F.F. preparations have less nucleic acid than PR8 preparations when expressed on a dry-weight basis (0.25%:0.9%) but about eight times more when the figures are compared on a virus particle basis. The value

Table 6. *Comparison of the base ratio of filamentous virus nucleic acid with that of an artificial mixture of equal amounts of spherical virus nucleic acid and potential host cell nucleic acid*

Source of nucleic acid	Nucleotide composition (as mole/100 mole nucleotide)				Adenine + uracil Guanine + cytosine
	Adenine	Guanine	Cytosine	Uracil	
Ryan filaments	21.2	25.3	26.2	27.3	0.94
1 part Ryan spheres + 1 part host cell	20.8	26.5	26.3	26.4	0.90

of the ratio for nucleic acid bases in Ryan F.F. preparations is quite different (0.9) from that found in Ryan Sup., being about half way between the latter value and that given by the RNA isolated from the uninfected host cell. A theoretical mixture of equal amounts of spherical virus type RNA and host cell type RNA yields values for the proportion of the individual bases which are very close to the value found in the RNA of Ryan F.F. (see Table 6). Support for such a concept is provided by two experimental results. First there is the finding that the nucleic acid associated with the soluble complement fixing antigen (CFA) isolated from Ryan F.F. gives a value for the proportion of nucleic acid bases which lies within the range given by the nucleic acid bases of A strain spherical viruses. Secondly, it was shown in Table 4 that the amounts of nucleic acid per unit of soluble CFA from PR8 and from Ryan F.F. are similar. (Nearly all the soluble CFA from Ryan F.F. will derive from filaments, the contribution from spherical particles being negligible.) There is also good agreement for the amount of soluble CFA released per AD of virus. As filaments in Ryan F.F. are 3 to 4 times more efficient as haemagglutinin than are PR8 spheres, it follows that these filaments have 3 to 4 times as much of the same type of nucleic acid as is present in PR8 spheres. Now Ryan F.F. preparations on a particle basis have 8 times as much nucleic acid as PR8 spheres. Nearly half of this is the 'spherical virus type'. By difference the remainder of the nucleic acid must have a composition close to that of unmodified host cell RNA. Is the host cell type RNA incorporated into the

structure of the filament? It would be very difficult to prove this but the evidence suggests that this is indeed the case. Treatment with high concentrations of ribonuclease did not alter the composition of the nucleic acid. Even after repurification, the breakdown products of filaments degraded by freezing and thawing contained the same amount of nucleic acid, with relatively unchanged properties, as the original Ryan F.F. preparation.

There are four further points which must be considered in any hypothesis concerning the formation of Ryan virus. (1) The spherical particles present in Ryan F. fluids are less efficient as haemagglutinin than are those particles present in preparations of PR8 or the mutant strain, Ryan Sph. viruses. (2) The particle weight of Ryan F. spheres is less than those of spheres in preparations of PR8 or Ryan Sph. (Ada *et al.* 1958). (3) The bulk of these

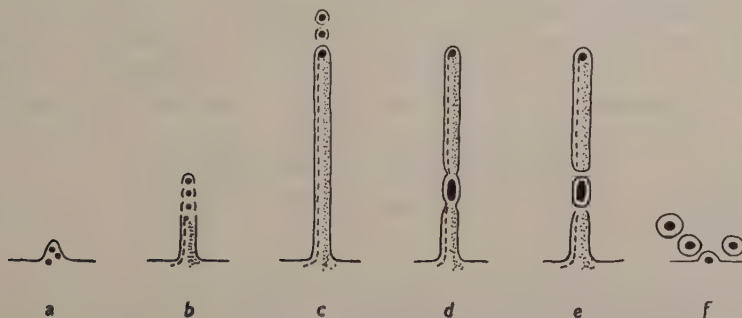


Fig. 1. A scheme illustrating the mode of formation of spherical and filamentary particles present in preparations of Ryan F. Spherical virus type RNA, ---●; host cell type RNA ■.

Ryan F. spheres are liberated into the allantoic fluid before the long filaments and hence may be formed before the latter. (4) Groups of linearly arranged spheres are often seen (unshaded micrograph) in Ryan F. preparations. These findings suggest that many of the spheres in Ryan F. fluids are not formed as distinct particles as is known to be the case in a strain such as PR8 virus (Morgan, Rose & Moore, 1956), i.e. the virus particles form at the surface of the membrane and separate cleanly as spherical bodies (Fig. 1*f*). Formation of Ryan F. spheres in this way would not readily explain their low efficiency as haemagglutinin or the smaller particle weight compared with spheres from Ryan Sph. preparations. A more attractive scheme is depicted in Fig. 1*a-e*. It is suggested that to a large extent the viral nucleoprotein is channelled into a filamentous structure (Fig. 1*a*); as the latter increases in length, the leading portion breaks up into particles (Fig. 1*b*) which, except for their smaller weight and a probable deficiency in surface components (including haemagglutinin), are similar to those in Ryan Sph. fluids. In some cases the filamentous particle continues to lengthen (Fig. 1*b, c*) and incorporation of spherical virus type RNA tapers off. Whether this decreased incorporation is gradual or sporadic, and whether inclusion of host cell type RNA is an attempt to remedy this deficiency, are unknown. There may be sectors which contain a

relative enrichment of spherical virus type RNA as suggested in Fig. 1*d*; breakage of such structures here would yield short filaments or spheres (Fig. 1*e*), many of which would be recovered in Ryan Sup. This interpretation implies that the filament breaks preferentially at those locations where there is an enrichment of viral type RNA. This is not unreasonable as there may be a constriction or weakening of the wall at these places, representing an abortive attempt by the infected cell to form spherical particles.

Does this scheme fit in with current ideas of the structure of filaments? If, for the purpose of discussion, it be assumed that filaments found in the allantoic fluid infected with different strains of virus are basically similar, the more important observations for which we must account are: (1) Filaments may have, particularly at one end, a large round structure called the Archetti body (Archetti, 1954) which may contain the virus nucleoprotein. (2) Sections of infected (Persia F. virus) allantois which were actively producing filaments show in the electron microscope no evidence of internal structure in filaments or structures corresponding to Archetti bodies; in comparable sections of membranes infected with a non-filamentary strain of virus, the virus spheres show an electron-dense core (Morgan, *et al.* 1956). On this basis it was suggested that the spherical form of the virus is the elementary infectious unit and that the filamentary form is largely or completely non-infective. (3) Filaments when treated with acid develop along their length rows of spheres which can be completely digested with trypsin. Spheres treated similarly reveal trypsin-resistant polygonal rings which have been identified as ribonucleoprotein (Valentine & Isaacs, 1957). (4) When subjected to procedures which break up the filament into approximately spherical particles, there is an increase in the haemagglutinin titre but no change in the infectivity titre (Donald & Isaacs, 1954; Burnet & Lind, 1957; this investigation).

These observations may be discussed in order. Classical Archetti bodies are rarely seen in Ryan F. preparations (Ada *et al.* 1958). The presence or absence of electron-dense material in virus structures has restricted meaning until it is correlated with the presence or absence of some chemical fraction. Valentine & Isaacs (1957) have carried out this further step by relating the appearance of specific structures visible in the electron microscope with their susceptibility to different reagents, including ribonuclease. Their evidence does not eliminate the presence of ribonucleoprotein along the length of the filament, but does show (i) that it is not present in as great a concentration as in spheres or (ii) that it is not in the same configuration which, when exposed to acid, causes it to be rearranged into a similar structure to that given by spheres. The final requirement, retention of infectivity following breaking up of the filament into smaller units, is not necessarily at variance with our scheme. It must be stressed that there is little information to indicate why an influenza virus particle may or may not infect the host cell. In the case of spheres it was found previously that 'incomplete' virus had a decreased nucleic acid content compared with the amount found in standard virus (Ada & Perry, 1955; Morzycki *et al.* 1956). On this basis the enhanced nucleic acid content of a filament may

be a contributing factor to the high infectivity. It is not inconceivable that a filament might break down into a number of smaller units containing varying amounts of nucleic acid but perhaps only one or two (presumably those containing sufficient RNA) are capable of inducing continuing infection (Ada, 1957) at the appropriate dilution. It is probable that in this case factors other than nucleic acid are involved.

The suggested mode of formation of spherical particles in cells infected with Ryan F. virus is different from that known for Persia F. virus where many spheres form as discrete particles (Morgan *et al.* 1956) and are as efficient as haemagglutinin as are spheres present in preparations of PR8 or Ryan Sph. viruses (Ada *et al.* 1958). That is, Persia F. virus is a strain where both types of virus production (filamentous and as discrete spheres) occur. It is unknown whether virus particles in the human host are produced as discrete spheres or by a filamentous process, similar to that postulated for Ryan F. virus. Attempts to examine in the electron microscope throat washings from humans suffering from influenza have so far been unsuccessful. The filamentous mode of virus formation may merely represent a transition stage in the adaptation from human host to egg host or it may characterize the production of virus in the human host. The apparent difference lies in the ability or otherwise to seal off the particle immediately after incorporation of the genetic material.

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A Study of *Mallomonas insignis* and *Mallomonas akrokomos*

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SUMMARY: Two species of *Mallomonas* (Chrysophyceae), *M. insignis* Penard and *M. akrokomos* Pascher ex Ruttner manuscript were investigated with optical and with electron microscopes. Carbon replicas have now made it possible to learn how the scales fit together in the armour and in particular how the tail, in each species, is constructed. Direct electron micrographs in conjunction with replicas have given much additional information about the scales. Developmental stages of *M. insignis* have been noted.

This paper describes two species of *Mallomonas* both of which are widely distributed in Europe. Both are common in southern England (Berkshire and Hampshire) and, in certain seasons, may become abundant. Both are easily recognizable with the low-power microscope and both prove to be exceedingly interesting when examined with the electron microscope, which clarifies several features that are puzzling with the optical microscope and reveals a wealth of unsuspected detail. *Mallomonas akrokomos* Pascher ex Ruttner manuscript and *M. insignis* Penard have nothing in common except that the posterior end of each is drawn out into a slender tail, but this tail is differently constructed in the two. Each species stands apart in the genus, showing no close affinity with any other in scale structure, while most of the numerous species of *Mallomonas* fall into natural groups with similar types of scale.

METHODS

The methods used in the examination of the *Mallomonas* spp. with optical and electron microscopes were described by Harris (1953) and Harris & Bradley (1957). Both direct and replica electron micrographs were used throughout the present work since they show different aspects of the scales. The direct electron micrograph shows the variation in the electron density of the scale substance, while the shadowed replica only shows the form of the exposed surface.

Free-hand drawings were made from living and dead material and annotated with dimensions; the final drawings were constructed from these. The development was studied from samples taken from two localities which both contained very numerous individuals.

MALLOMONAS INSIGNIS

History. *Mallomonas insignis* was first described by Penard (1919) in a very careful and detailed paper; his description was discussed in Conrad's monograph (1933) and briefly summarized by Huber-Pestalozzi (1941);

certain aspects were further elucidated by Bourrelly (1951). While I am in agreement with much that has been published by these authors about this organism I do not accept all their interpretations and shall discuss these differences later.

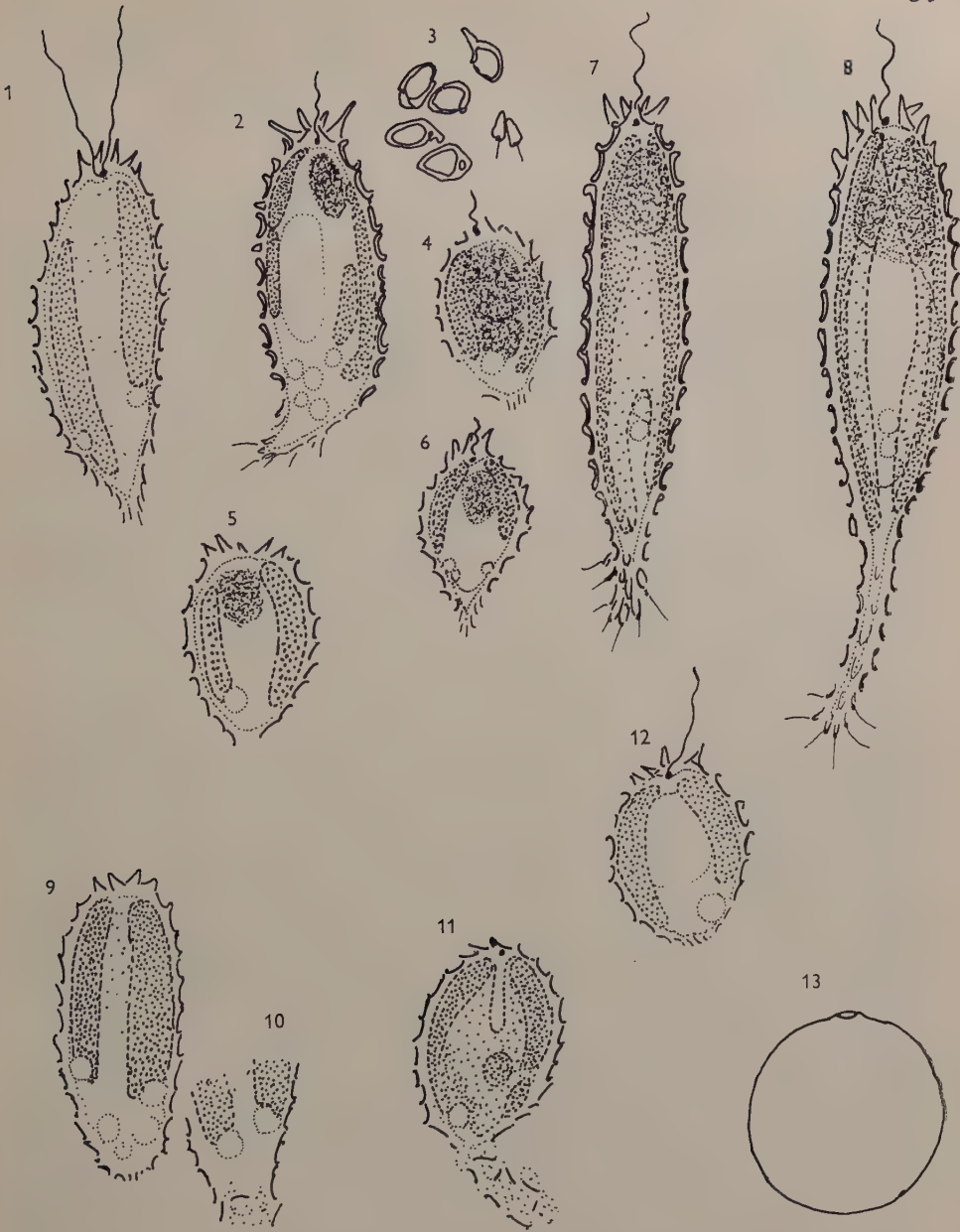
Occurrence. *Mallomonas insignis* has been found in ponds, ditches and flooded fields on agricultural land and also in pools and lakes on acid peat. It may be found from time to time between late November and April but, like all species of *Mallomonas*, it tends to be both brief and erratic in its occurrence. In suitable seasons, especially when winter floods begin to dry up, it may be found in very large numbers, but in some seasons it is only rarely seen. Cysts have been found in April.

Description of Mallomonas insignis as seen with the optical microscope

Mallomonas insignis is a long spindle-shaped organism bluntly pointed in front and tapering to a tail of varying length at the rear. The flagellum varies from about one-quarter to three-quarters of the body length. A large conspicuous nucleus is present at the anterior end and there is frequently a leucosyn body behind this. The form of the chromatophore is usually obscured by the strongly refractive corrugations of the armour, but whenever I have been able to see it, it has been single and divided into two lobes by a cleft at the anterior and posterior ends. The anterior cleft is always short and usually conspicuous. The posterior cleft may be short as in Fig. 7 or may be so long that it nearly meets the anterior cleft, as in Fig. 8. Contractile vacuoles are usually present in the posterior cleft. Sometimes, presumably when the nucleus is about to divide, the chromatophore shows several lobes and its structure is obscure. The surface of the cell is covered with a rather rigid armour of thick concave silica scales which are conspicuous in the living cell. The anterior scales bear short prominent spines, the middle scales are spineless, while the scales on the tail are quite small and bear slender spines which are not always visible on the living organism. A fine strand of protoplasm runs down the whole length of the tail. *M. insignis* is sluggish, sometimes swimming rather slowly but often remaining still for long periods with a gently waving flagellum. Cysts (Fig. 13) are nearly spherical, smooth with a posterior pore which often protrudes slightly, size $14-28\mu \times 25-30\mu$.

Development

Figures 1, 2, 4, -6 and 9-12 illustrate stages recognized in the development of *Mallomonas insignis*. The anterior part of the cell in all organisms examined had scales resembling those of a mature cell, but the middle part was often only lightly armoured while the tail was not developed or was short and feebly armoured. I have not been able to see an individual without a tail develop a fully-formed tail, but I have often watched the protoplasm extrude through the posterior end as though to form a tail. Since this extrusion was limited and the contractile vacuole and flagellum continued to function normally, I concluded that the extrusion was part of normal development and not due to the cell being damaged or moribund. Figures 9 and 10 show a change.



Figs. 1-13. *Mallomonas insignis*. All figures are $\times 1000$. 1. Dividing individual with two flagella, posterior end immature. 2. Individual showing four chromatophore lobes and numerous contractile vacuoles, posterior end immature. 3. Group of scales, two with spines from anterior end, three without spines from middle and two small ones with spines from tail. 4. Very early stage showing two nuclei, some scales peeling off, others missing, delicate parallel scales where tail will develop. 5. Very early stage without flagellum showing a gap in the armour where the tail will develop. 6. Very early stage with delicate immature tail already formed. 7. Mature individual with short tail showing a short posterior cleft in the chromatophore. 8. Mature individual with long tail showing very long posterior cleft in the chromatophore. 9. Early stage without flagellum, no tail present but group of parallel scales at rear, numerous contractile vacuoles present in rear part of cell. 10. Rear end of same individual some minutes later showing cytoplasm extruding. 11. Early stage showing long protoplasmic extrusion at rear. 12. Very early stage showing delicate parallel scales at rear. 13. Cyst.

taking *c.* 10 min.; in the rear end of a single cell, which I watched. Figure 11 shows a cell with an unusually long extrusion of protoplasm which showed no change and no signs of dying during *c.* 10 min. while I watched it.

Description of Mallomonas insignis as seen with the electron microscope

(Plates 1 and 2)

The whole cell is seen to be covered with scales which are elaborately sculptured on the outer side, flat or slightly concave and relatively smooth on the inner. They vary progressively from the anterior to the posterior end of the organism but the same basic pattern can be traced through all. No dome and no bristles are present but a shield and flange can be distinguished. The shield is bounded on the free side by a broad thickened ridge usually covered by papillae. A hollow spine arises from this ridge in some scales. There is a raised fold where the shield joins the flange, and in many scales this is somewhat hooded, overhanging the shield. The central area of the shield is thin and may show a few groups of papillae. The part of the flange joining the shield is fairly thin and bounded by a thickened rim rising steeply and often hooded, while it slopes gradually towards the margin. Direct electronmicrographs of some scales (Pl. 1, fig. 6) show minute perforations over the whole scale, but in others these perforations are not apparent.

The extreme anterior scales are a little smaller than the succeeding scales and bear stout hollow spines, ridged at the base and bearing a characteristic bent tip (Pl. 1, figs. 1, 2). The next scales have smaller spines (Pl. 2, fig. 7) and those following have no spine at all (Pl. 1, fig. 3; Pl. 2, fig. 8). After this there is not much change, except that the scales become slightly broader and rounder (Pl. 2, fig. 10) until the cell narrows towards the tail. Here the scales become a good deal smaller, asymmetrical and somewhat varied in detail (Pl. 1, figs. 5, 6; Pl. 2, fig. 14). Some of these have a short spine with a bent tip (Pl. 1, fig. 3). They are intermediate in shape and size between the scales in front and those of the tail. On the tail the scales are once again symmetrical, smaller, less heavily sculptured and differing in the shape of the sculpture from the forward scales. They have slender spines which vary in length and are longest at the end of the tail. The scales at the anterior end of the cell point forwards, those in the middle have their long axis across the cell while those on the tail overlap so that the free end, with the spine, points backwards. Pl. 1, fig. 4, shows a replica from about the middle of a cell in which the scale armour has not been disturbed. The scales lie in rows across the cell and are so placed that the flange is always hidden by the overlap of the neighbouring scales. At the top right-hand side, where a scale has broken away, a flange is visible. The scale arrangement is the same as in other species which have been studied, e.g. *Mallomonas leboimii*, *M. lichenensis* and *M. coronata* and others of the same groups (Harris & Bradley, 1957). Each of these species has a flange varying in form and in breadth, and in each case it is covered by neighbouring scales in the unbroken armour. Although *M. insignis* stands apart from other species in the features it shows under the optical microscope, its

scales are normally arranged. Plate 2, fig. 11, shows part of the tip of the tail with its scales overlapping so that the spines point backwards. The spines are of varying length.

Discussion

On the whole this account of *Mallomonas insignis* corroborates and amplifies Penard's description but my interpretation of the tail extremity differs from his. He says that the cytoplasm comes out at the end of the tail and forms a number of adhesive threads which serve to fix the cell to a substratum. These he shows in his figure, which is reproduced by Conrad (1933; fig. 22*b*) and by Huber-Pestalozzi (1941; taf. XXII, fig. 13I). Penard says that these filaments are silica threads coated with glutinous or mucilaginous material and that they move in currents of water. As we have seen these 'filaments' are slender spines, outgrowths of the scales.

The description of the chromatophore by previous authors, as two structures, rather than a single bilobed one differs from my interpretation. Bourrelly (1951) first demonstrated the bilobed structure of a *Mallomonas* chromatophore in *M. coronata* Perman and Vinnikova (syn. *M. doignonii* Bourrelly). Harris (1953) and Harris & Bradley (1957) showed the same type of structure in a number of other species. Although the structure of the *M. insignis* chromatophore is particularly difficult to see, I believe it to be of the same nature as those of other species.

Penard suggested that *Mallomonas insignis* might be merely a variety of *M. pulcherima* Stokes (1888), at that time known only from America. (I have not been able to see Stokes's paper.) This species is now known also from Europe and the figures given by Stokes for the American specimens and by Conrad for the European ones, while they agree with each other, differ considerably from *M. insignis*. I think, therefore, that the two species have rightly been kept apart.

Mallomonas torulosa Kisselew (1931) must, I consider, be *M. insignis*. The drawing suggests *M. insignis* at a stage when its tail is not fully developed. No indication of the scales is given but its size, $81 \times 15 \mu$ is the same as that of *M. insignis*. *M. mesolepis* Skuja (Huber-Pestalozzi, 1941) is, I believe, a synonym of *M. insignis*. The size and appearance are that of *M. insignis* with a somewhat immature tail. The scales are rhomboidal rather than oval but, though the scales of *M. insignis* are oval, the disposition of the thickening often makes them appear somewhat rhomboidal under the optical microscope and they certainly look rhomboidal when seen on the living organism.

MALLOMONAS AKROKOMOS PASCHER EX RUTTNER MANUSCRIPT

History. *Mallomonas akrokomos* was described by Ruttner in a manuscript which was first published by Pascher (1913). It is usually known as *M. akrokomos* Ruttner, but the correct name would seem to be that given above. It was later described by Swirenko (1924), Schiller (1926), Conrad (1927), Krieger (1930), Woloszynska (1939) and Nygaard (1949). Asmund (1956) was the first to study the isolated scales which she figured as seen with the optical

and the electron microscope. Both Conrad (1933) and Huber-Pestalozzi (1941) published descriptions and figures and these are probably the most easily obtainable references. The descriptions of all these authors agree with each other on most points. A few varieties have been described and these will be discussed later.

Occurrence. *Mallomonas akrokomos* is by far the commonest species of *Mallomonas* in Hampshire and Berkshire. It occurs in a great variety of localities such as ponds, lakes, ditches and flood waters, both on acid peat and on agricultural land. I have found it in each month of the year except September, but it is rare in July and August and certainly commonest in winter and early spring. Cysts have been found from December till April. It is a species which thrives in cold weather and may become abundant under ice. In spite of its commonness it is sporadic and one can rarely count on finding it at any given time or place. It has been reported from Austria, Germany, Belgium, Russia, Poland and Denmark, but only in cold weather, whereas in Britain though it is commonest in cold weather it also occurs in warm weather.

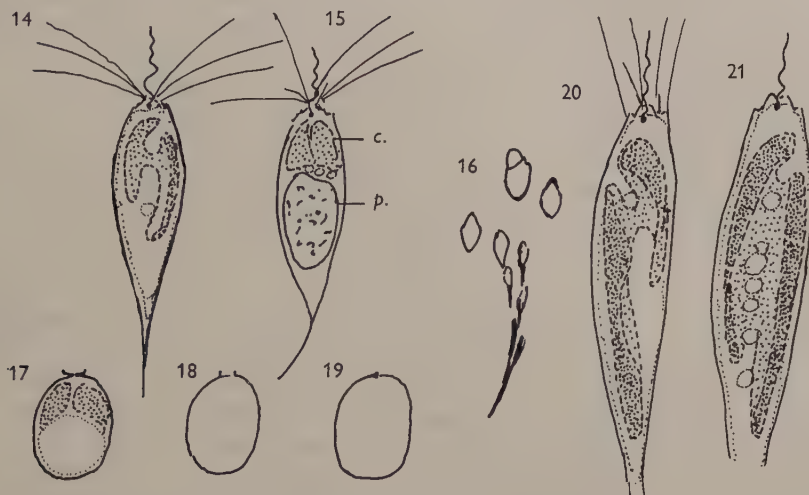
Description of Mallomonas akrokomos as seen with the optical microscope

The organism is spindle-shaped tapering to a slender tail which may be straight or bent; its length is 20–60 μ or occasionally more, and the breadth 5–15 μ . A group of bristles usually occurs at the anterior end of the cell; frequently eight bristles are present, four or five being long and four or three being short. The number of long bristles may, however, vary from three to six and the short ones from none to four. While I have seen individuals with no bristles at all or with long bristles only, I have not seen any with short bristles only. The bristles of the living organism are more or less curved especially when the individual is swimming and they may be directed forwards or outwards or some forwards and some outwards. The bristles of a dead or fixed individual appear straighter, but here also there is a good deal of variation. With the optical microscope the bristles look smooth. The long bristles vary from 19 to 35 μ and the short ones from 8 to 15 μ . The flagellum is commonly half body-length but may be considerably longer. The chromatophore is single, usually divided into two lobes by posterior and anterior clefts which nearly meet. There are often contractile vacuoles present in both posterior and anterior clefts. Sometimes no cleft is clearly visible and the division of the chromatophore seems to consist of a string of contractile vacuoles (Fig. 21). The lobes are usually obliquely placed and may be very unequal in size. The cytoplasm does not pass down the tail to its tip (Fig. 14). Movement is very fast. The cyst is oval and smooth, its pore may be surrounded by a narrow or broad rim or none at all, and may be posterior or anterior. I have seen cysts of both types in the same sample of water.

The scales as seen on the unbroken cell may, in some conditions, appear like small triangles, such as were described by Conrad. Isolated scales look like delicate oval or slightly pointed plates, and become narrower as they approach the tail. The bristle-bearing scales, which are at the extreme anterior end, are

oval with a clearly visible dome. The tail itself is usually seen as a single rod, though occasionally two rods may be seen lying side by side.

I have many times watched *Mallomonas akrokomos* dividing. Division was longitudinal, starting with the flagellum and passing from the anterior to the posterior end of the cell. When it was complete the two daughter cells swam away from each other.



Figs. 14-21. *Mallomonas akrokomos*. Figs. 14-19, $\times 1,000$; Figs. 20-21, $\times 2,000$. 14. Individual with bristles in position usual when swimming. 15. Cell infected by a parasite, (cf. Harris, 1953), swimming (c.=remains of chromatophores, p.=parasite). Two short bristles present and one forward pointing long bristle. 16. Group of scales including anterior middle and tail scales. 17-19. Cysts showing variation of pore. 20. Cell with normal chromatophore, short and long bristles showing shape of anterior end of cell. 21. Cell without bristles, chromatophore divided by a string of contractile vacuoles, shows shape of the anterior end. In Figs. 20, 21 the tail is omitted.

Description of Mallomonas akrokomos as seen with the electron microscope

(Plates 3 and 4)

The scales of *Mallomonas akrokomos* as seen in the electron microscope are rather different from those of most other species of *Mallomonas* and it is not possible to apply the terms shield and flange, though a dome is present on the bristle-bearing scales. All the scales are elongated and form thin slightly convex plates of silica and all show a more or less oval patch of close perforations near their posterior end, but other details vary in scales from different parts of the cell. Only the two anterior rows have bristle-bearing domes. These scales are also distinguished by having longitudinal ribs a little inside the thickened margin, separated from the margin by a row of perforations. These scales are of two sizes, the ones next the flagellum being smaller than the succeeding ones. None of the rest of the scales have a dome, and all, including the tail scales, have a toothed anterior margin. The scales over the main part of the cell are somewhat oval, bluntly-pointed at each end and becoming narrower further from the anterior end. These scales are thickened at the

posterior end and have a thickened posterior margin, inside which is a single row of perforations. As the cell narrows towards the tail the scales become increasingly narrow and convex and change somewhat in shape. They become rounded in front and taper towards their thickened posterior end. Usually these scales are somewhat shorter than those further forward. The last two scales form the tail; their anterior part is strongly concave and they taper to a fine point and are two or three times the length of the previous scales. Thickened ridges extend down the converging sides and join at the end. These two scales fit closely together forming an elongated tube, the upper part of which overlaps the scales next in front (Pl. 3, fig. 20; Pl. 4, fig. 23).

The bristles are similar to each other except in length. They are toothed and have a club-shaped foot which is more thickened than that found in most species. Some are nearly straight and some distinctly curved. Plate 4, fig. 21, shows the group of bristles belonging to a single cell.

Discussion

Mallomonas akrokomos has been placed by Conrad (1927) in a group by itself called 'triangulares' as distinguished by its 'triangular' scales. I think that the triangles Conrad saw were just the exposed ends of the scales. *M. akrokomos* does, however, stand by itself, for no other species has elongated scales placed longitudinally. Many species have oval scales but they are placed in oblique or transverse rows and overlap, to a greater or less extent, laterally. In *M. akrokomos* the very considerable overlap is always on the longitudinal axis. In addition, no other species has a tail made of two much elongated scales as in *M. akrokomos*. The posterior end is usually composed of smaller scales than the rest of the cell.

The chromatophore of *Mallomonas akrokomos* is interesting. In all the species of *Mallomonas* that I know the chromatophore is divided into two lobes by an anterior and posterior cleft. A contractile vacuole may sometimes be seen in the anterior cleft of some species and one or more are often seen in the posterior cleft of many species. I have never seen in any other species, what is sometimes met with in the chromatophore of *M. akrokomos*, namely, a chromatophore divided into two lobes only by a string of contractile vacuoles.

The longitudinal division of the cells described here differs from that described by Conrad (1927). He stated that the contents of the mother cell divided and that later the two daughter cells escaped from the armour of the mother cell. Conrad's type of division corresponds with that described by Carter (1937) for *Mallomonas anglica*, but I have not yet observed it in *M. akrokomos*.

A number of varieties of *Mallomonas akrokomos* have been described. *M. akrokomos* var. *parvula* Conrad (1927) differs from the type by its considerably smaller size, 8–12 μ long \times 2–3 μ broad, and by having only four or fewer bristles. The description is perfectly clear and this is probably rightly regarded as a variety. It is possible, however, that it may be found to be only a developmental stage of *M. akrokomos* corresponding to the stage called

'small oval' in the study of *M. intermedia* var. *gesticulans* (Harris, 1953). If a good sample of this variety should be found it would be interesting to follow its development.

Woloszynska (1939) described some Polish specimens as *Mallomonas akrokomos* forma *Tatrica*. She considered that they differed from the type in having shorter and straighter bristles, up to 15μ long. In the material I examined most closely the bristles were up to 35μ long, though they were often shorter. I do not know the significance of these differences of maximum length. Considerable variation in curvature may be seen in a single individual (Pl. 4, fig. 21). Woloszynska was the first person to observe that the scales are really oval and that the bristles are usually of two lengths in one cell, though their numbers are very inconstant.

Mallomonas akrokomos is sometimes attacked by a parasite which, after penetrating the cell, usually near the rear, gradually consumes and replaces most of the chromatophore. The front of the host cell, however, remains active and apparently uninfected. The parasite forms a cyst while still inside the cell armour (Fig. 15). The parasite closely resembles that described more fully for *M. teilingii* (Harris, 1953), the spore of which had earlier been regarded as a distinct species of *Mallomonas*.

I should like to thank Dr D. E. Bradley of the Research Laboratory, Associated Electrical Industries, Aldermaston, Berkshire, for taking the electron micrographs, and Dr T. E. Allibone, F.R.S., Director of A.E.I. Research Laboratory, for permitting this.

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EXPLANATION OF PLATES

PLATE 1

Mallomonas insignis. Replicas. $\times 6000$

- Fig. 1. Inner side of anterior scale with spine, hollow at base.
- Fig. 2. Outer side of anterior scale with spine.
- Fig. 3. Outer side of scale from near anterior end of cell.
- Fig. 4. Outer side of undisturbed scale armour from about middle of cell.
- Figs. 5, 6. Outer side of scales from part of cell which narrows towards the tail.

PLATE 2

Mallomonas insignis: Figs. 7, 8 and 10 direct electron micrographs; figs. 9, 11-14, replicas. All $\times 6000$, except fig. 4, which is $\times 8000$.

- Fig. 7. Anterior or near anterior scale, with small spine.
- Fig. 8. Scale from near anterior end of cell.
- Fig. 9. Outer side of scale from front part of tail, with short spine.
- Fig. 10. Scale from middle of cell showing perforations.
- Fig. 11. End of tail showing variation in bristle length and backward pointing scales.
- Fig. 12. Outer side of tail scale.
- Fig. 13. Inner side of tail scale showing hollow in base of spine.
- Fig. 14. Inner side of scale from part of cell which narrows towards the tail (compare scale Pl. 1, fig. 5).

PLATE 3

Mallomonas akrokomos. Replicas. All $\times 8000$

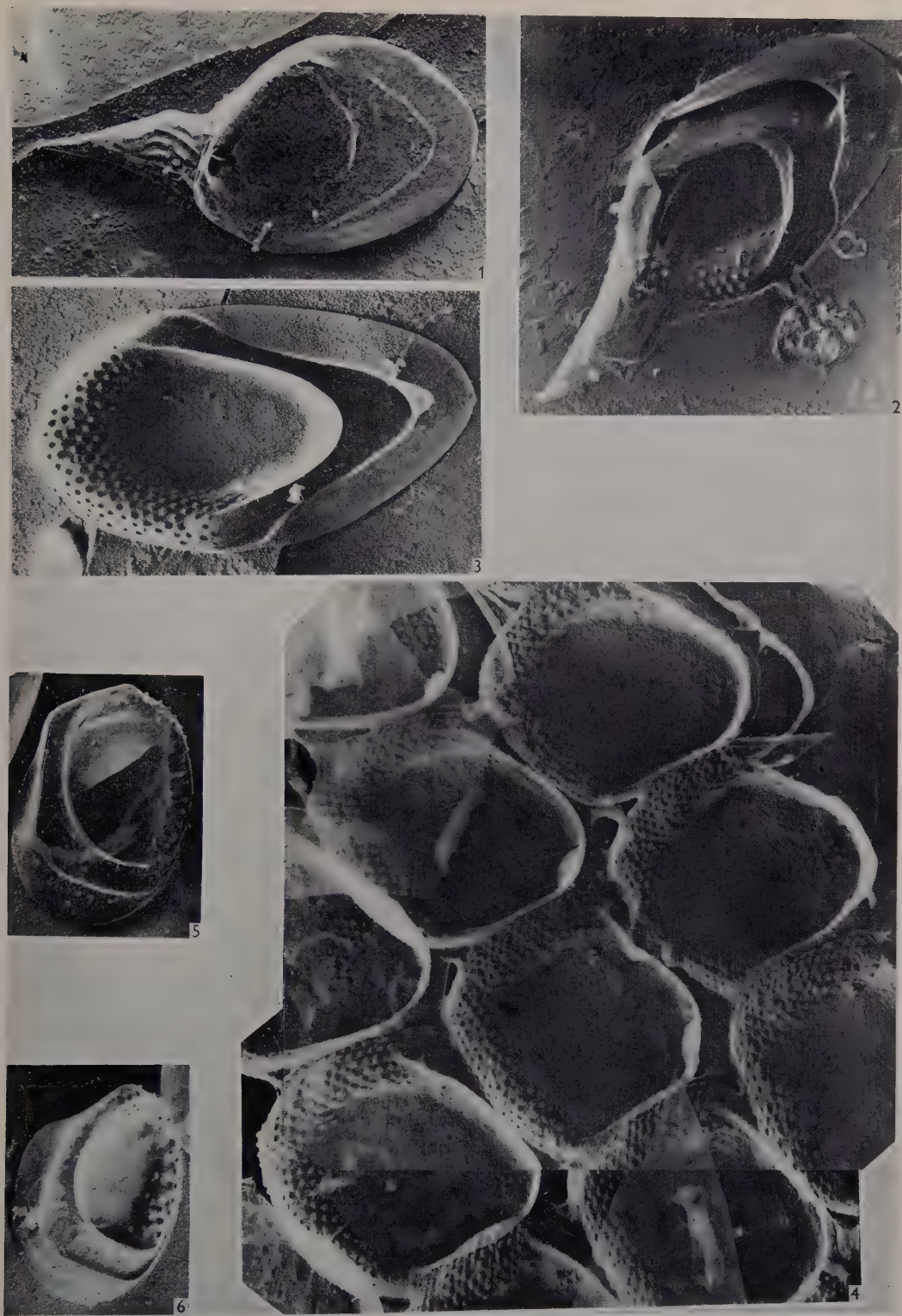
- Fig. 15. Inside of group of anterior scales of two sizes and some bristle bases.
- Fig. 16. Outside of two small anterior scales.
- Fig. 17. Outer side of scale from near posterior end of cell showing regions of perforations and thickened margin (compare Pl. 4, fig. 25).
- Fig. 18. Scale armour just in front of tail showing scales in longitudinal rows.
- Fig. 19. Group of scales from near tail showing serrated forward margins.
- Fig. 20. Tail and scales just in front of it, one tail scale facing upwards with thickened margins which meet at extreme end.

PLATE 4

Mallomonas akrokomos. Direct electron micrographs. $\times 8000$.

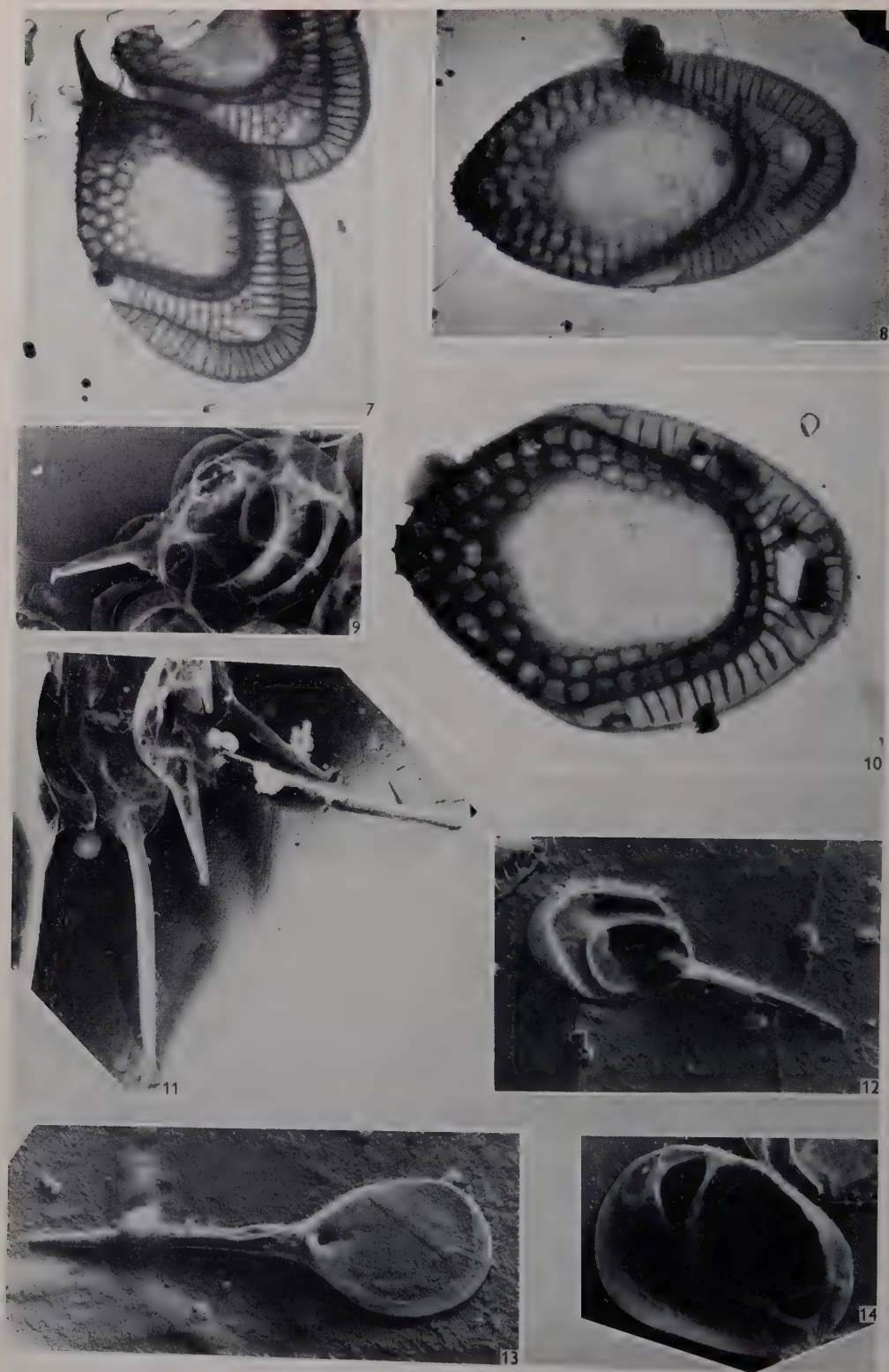
- Fig. 21. Group of eight bristles of varying length and curvature.
- Fig. 22. Two anterior scales of different sizes, showing perforations.
- Fig. 23. Tail, showing the two scales slightly separated and the base of scales just in front.
- Fig. 24. Group of scales from different parts of the cell but all fairly near the middle.
- Fig. 25. Scale from near anterior end of cell.
- Fig. 26. Scale from near tail.

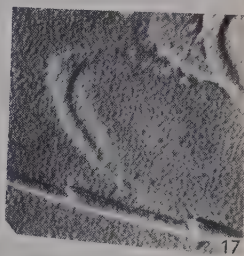
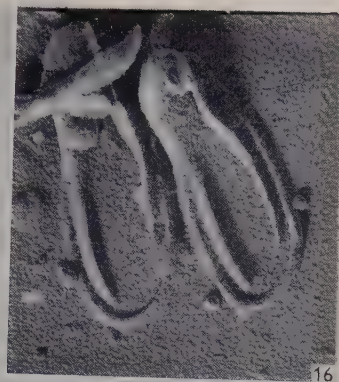
(Received 28 December 1957)



K. HARRIS—ELECTRON MICROSCOPY OF MALLOMONAS. PLATE 1

(Facing p. 64)







MASON, D. J. & POWELSON, D. (1958). *J. gen. Microbiol.* **19**, 65-70

Lysis of *Myxococcus xanthus*

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SUMMARY: *Myxococcus xanthus* lyses not only in old cultures but also when suspended in solutions of monovalent cation salts. This lysis is slow at 37° and lower temperatures, but is very rapid at 45° and 60°. Organisms in distilled water do not lyse at any temperature. Lysis is more rapid at high pH values, in broth or in solutions of monovalent cation salts; it is optimal in c. 0.03M solutions of the salts. During lysis the cell wall weakens and empty spherical protuberances develop at the ends or in the middle of the organism. Finally, the dense material of the protoplasm disappears except for an occasional granule, and only single or paired spherical ghosts remain.

Studies of the lysis of bacteria have been important in contributing to knowledge about cell walls. Much significant information has been obtained through the introduction of enzymes as lytic agents (Salton, 1953; Salton 1956; Weibull, 1956), but the 'autolysis' of bacteria has also been an interesting aspect of cell lysis (Nomura & Hosoda, 1956; Mitchell & Moyle, 1956, 1957; Strange & Dark, 1957). During a study of the cell wall of the myxobacteria, it was found that the organisms lysed readily. This paper discusses some conditions favouring lysis and demonstrates the morphology of the lysing cells.

METHODS

Myxococcus xanthus was used as a representative myxobacterial organism. Stock cultures were grown for 7 days at 30° on a modified Noren's medium (Noren, 1952), solidified 1.5% (w/v) agar. The liquid medium contained (w/v) trypticase (Baltimore Biological Laboratory, U.S.A.), 0.5; K₂HPO₄, 0.25; NaCl, 0.1; MgSO₄·7H₂O, 0.01; traces of Ca(NO₃)₂, FeSO₄, MnSO₄. The pH value of the medium after autoclaving was 7.2-7.4. All of the organisms used in the experiments were grown in 150 ml. volumes of the trypticase+salts medium in 1 litre flasks at 27°; the flasks were shaken at 100 strokes (2.25 in. throw)/min. A strain of *M. xanthus* was used which grew diffusely in the liquid medium. This strain did not spread on agar solidified medium, but it retained the ability to form fruiting bodies and microcysts (J. Adye, 1958 private communication). As source of inoculum, 5 ml. of culture were transferred to flasks of medium every 48 hr. The organisms used for experiments on lysis were from 48 to 60 hr. cultures, in the early part of the stationary phase of growth. They were washed with distilled water three times by centrifugation and finally resuspended in distilled water.

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Changes in optical density were used to follow lysis and to obtain growth curves. These changes were observed with a Coleman model 14 spectrophotometer at 610 m μ . In tests for lysis, 3 ml. suspension of organisms were added to 3 ml. of solution under test. The optical density (OD) of this mixture was about 0.8–0.9 scale reading unit. The readings on duplicate tubes throughout the test never varied more than 0.02–0.03 OD unit. Results are expressed as the average of the duplicates.

The morphological changes which took place during lysis were followed by phase contrast and electron microscopy. Wet mounts of lysing organisms were observed with a Spencer $\times 97$ oil immersion bright high phase contrast objective. Photographs were taken at $\times 1500$ on Adox KB 14 film. Specimens were also prepared for ultra-thin sectioning. Non lysing or lysing organisms were fixed for 2 hr. at 25° in OsO₄ (2 %, w/v) solution in pH 7.3 veronal buffer. The organisms were dehydrated in ethanol and embedded in *n*-butyl methacrylate+methyl methacrylate (9:1). Sections were cut with a Porter-Blum microtome and examined in a Phillips electron microscope.

RESULTS

Figure 1 shows a typical growth curve of *Myxococcus xanthus* in the modified Noren's liquid medium. After the cultures reached the stationary growth phase, there was a gradual lysis. During this time, the pH value of the culture medium might go as high as pH 8.5. Similar lysis occurred in solutions of NaCl, and was much more rapid at 45° than at 27°. Figure 2 shows the rate and extent of lysis of washed organisms in different concentrations of NaCl at 45°, they lysed in almost all concentrations of NaCl tested, but the rate optimum was around 0.03 M. Figure 3 compares the lysis of these organisms in the liquid medium in which they have been grown with lysis in 0.04 M-NaCl. There was no lysis in distilled water. The organisms also lysed in 0.03 M solutions of KBr, NaF, KCl, K₂SO₄, Na acetate, NH₄Cl, (NH₄)₂SO₄ and NH₄ acetate. The rate and extent of lysis varied with the anion in salts of monovalent cations, as shown in Fig. 4 for ammonium salts. However, the addition of divalent cation salts to suspensions did not result in lysis; solutions of 0.03 M-FeSO₄, MgCl₂ and CaCl₂, and 0.01 to 0.1 M-MgSO₄ were tested. MgSO₄ (0.0025 M) added to the liquid medium completely inhibited lysis; CaCl₂ did not. Takahashi & Gibbons (1957) reported that small amounts of divalent cations helped a halophilic bacterium (*Micrococcus denitrificans*) maintain its cell wall and allowed it to survive in less than the normal minimum level of sodium chloride.

The rate and extent of lysis were determined at different temperatures in the broth from the stationary growth phase (Fig. 5); the optical density (OD) changes of suspensions in water are also shown. The OD of water suspensions increased as the temperature increased; in contrast, the OD of suspensions in the liquid medium decreased. The rate and extent of lysis in both was strikingly greater at 45° and 60° than at 37°. Similar results were obtained when the organisms were lysed in 0.03 M-NaCl at these temperatures.

Lysis was affected by the pH value of the suspending solution. Two ml. of a washed suspension were added to 4 ml. 0.05M-veronal buffer (0.03M final concentration). The veronal buffer (pH 7.0-9.0) was made by adjusting the

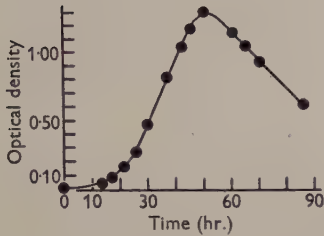


Fig. 1

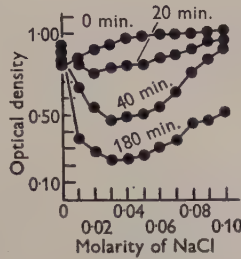


Fig. 2

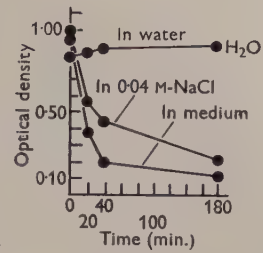


Fig. 3

Fig. 1. Growth curve, by optical density, of *Myxococcus xanthus* (disperse growing strain). Shaken in trypticase+salts medium at 27°.

Fig. 2. Lysis of *M. xanthus* suspended in NaCl solutions at 45°.

Fig. 3. Rate of lysis of *M. xanthus* at 45° in water, 0.04M-NaCl or medium.

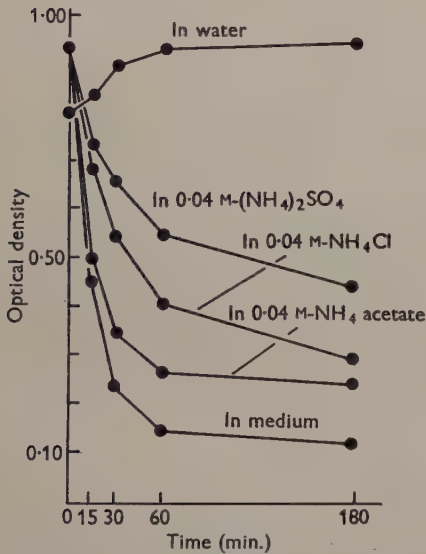


Fig. 4

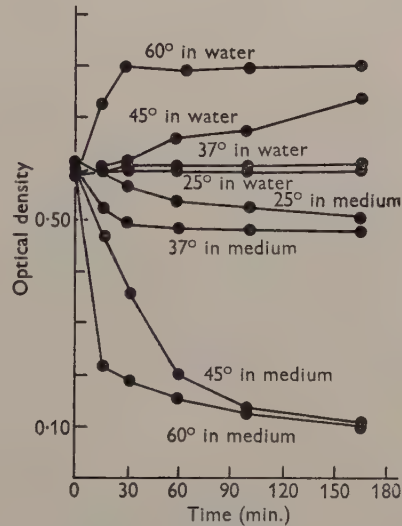


Fig. 5

Fig. 4. Lysis of *M. xanthus* at 45° in 0.04M-NH₄Cl, (NH₄)₂SO₄, NH₄ acetate: water or medium and broth.

Fig. 5. Lysis of *M. xanthus* in water or medium at different temperatures.

pH of 0.1M-Na veronal to the desired values with 0.1M-HCl; then the buffer was diluted to 0.05M-veronal. The rate and extent of lysis increased as the pH increased and lysis was almost instantaneous in other solutions with pH > 9.5, even at room temperature.

Morphology of the organisms during lysis

Myxococcus xanthus grew as a long slender rod in the trypticase + salts medium. The organisms were slightly less than 0.5μ in diameter and $5-10\mu$ long. Thin sections of organisms from 48 hr. cultures showed that division occurred by a 'pinching-in' at the centre of the organism (Pl. 1, fig. 1). The organisms were surrounded by a wall which had an electron dense layer *c.* 75\AA . on each side of a non-electron dense layer about 100\AA thick. In non-lysing vegetative organisms these layers were never separated even when the organisms were poorly fixed.

Lysis was followed with phase-contrast microscopy. Plate 2, fig. 2, shows growing unaffected vegetative organisms from a 48 hr. culture. An organism (48 hr.) beginning to lyse developed a 'bulging' that was very characteristic of this lysis (Pl. 2, fig. 3). When the organisms were completely lysed, there was nothing visible except spherical ghosts in the suspension (Pl. 2, fig. 4). A thin section of lysing organisms (48 hr.) is shown in Pl. 2, fig. 5, while an air-dried preparation of lysing organisms is shown in Pl. 2, fig. 6. During lysis the cell walls seemed to weaken and bulbs formed usually at the ends of the organisms. When the lytic process was complete, spherical ghosts were left. Air-dried ghosts (Pl. 3, fig. 7) bore some resemblance to isolated cell walls (Mason, 1958). Thin sections of these ghosts suggested the presence of internal 'membranes' (Pl. 3, fig. 8).

The lysis of organisms from 38 to 60 hr. cultures by solutions of NaCl was not prevented by high concentrations of sucrose. However, Adye (1958) prevented lysis in medium from the stationary growth phase by adding sucrose or various gums. Mason observed that organisms from 12 to 20 hr. cultures rounded up without lysis when incubated on 0.5 M -sucrose at 30° or after a day or so at 4° . The changes which occurred with 12 hr. organisms during a period of 90–120 min. at 30° are illustrated by Pl. 3, figs. 9–12. Though the organisms remained dense they were not 'protoplasts' since they did not lyse when the suspension was diluted 1/10 with distilled water. Thin sections of these organisms indicated that they might be highly vacuolated (Pl. 3, fig. 13). Although these round cells were not viable by our tests, Adye (1958) demonstrated viability when special methods were used.

DISCUSSION

While vegetative cells of *Myxococcus xanthus* lose the usual rod shape perhaps more easily than do eubacterial bacilli, the changes which occur appear to be similar in nature. It has been suggested (Park & Strominger, 1957) that in eubacteria changes in the shape occur under conditions that seem to effect a loss or weakening of structural elements of the cell wall. The 'rabbit ears' and other oddly shaped cells, including round bodies, seen in suspensions of *Escherichia coli* during lysis promoted by lysozyme, penicillin or other agents, also appear during lysis of myxobacteria in NaCl solutions. Previously, the existence of a definite cell wall in myxobacteria has been questioned (e.g. van Iterson, 1947; Klieneberger-Nobel, 1948). On the other hand, different types of round bodies of myxobacteria other than microcysts have frequently been

observed. Stanier (1947) reported that almost coccoid cells were present in cultures of *Cytophaga johnsonii*. Bachmann (1955) observed that 'small round bodies of low refractility' occurred chiefly in old cultures. Gray (1957) found two types of round cells, a small 'spheroid' and a larger cell that might be a 'spheroid' with layers of slime. The data we have presented indicate that myxobacteria have a structure that is morphologically identical with the cell walls of certain eubacteria. While we found that *Escherichia coli* and *Bacillus cereus* did not lyse or round up in the NaCl solutions, Mitchell & Moyle (1956, 1957) observed that rapidly growing *Staphylococcus aureus* and *E. coli* became osmotically fragile in NaCl solutions and were stabilized by high concentrations of sugar. They demonstrated an autolytic system in the cell walls of *S. aureus*. It is possible that an autolytic enzyme or factor functions similarly in myxobacteria. One might be led to believe that this autolytic system and the protease in myxobacteria that is known to lyse eubacteria (their protoplasts but not cell walls) act synergistically to clear myxobacteria suspensions, especially when certain environmental factors hasten the death of the organisms; for example, the extent as well as the rate of lysis of the myxobacteria in the liquid medium and in NaCl solutions was much greater at 45° and 60° than at 37°. However, the absence of lysis in distilled water at these temperatures needs to be explained. If the organisms are being killed by heat the protease alone should bring about some lysis in water suspensions. Since this did not occur, perhaps we should consider the possibility that the protease is liberated from an inactive complex by the attack of an autolytic enzyme on its specific substrate. Then, only when conditions favour the action of the autolytic enzyme, can the protease hydrolyse the protein of the protoplasm.

The morphology of the boundary of a ghost of *Myxococcus xanthus* (Pl. 2, fig. 5; Pl. 3, fig. 8) suggests that most of the original cell wall remained after lysis. Mitchell & Moyle (1956, 1957) showed that eubacteria may become osmotically fragile without loss of much structural cell-wall material. By way of contrast again, it may be pointed out that round bodies of *M. xanthus* produced in 0.5M-sucrose (Pl. 3, fig. 12) are not osmotically fragile; we should like to know what stabilizes these cells. Since *M. xanthus* produces spherical microcysts on agar surfaces during fruiting-body formation, these round bodies may represent an aborted form of microcysts.

We wish to thank Mr J. G. Holt for the culture of *Myxococcus xanthus* which he isolated and identified. A predoctoral fellowship to D. J. Mason from the National Institutes of Health was appreciated. The discussions with Mr J. Adye, Mr J. G. Holt, and Miss Lois Nellis, who are also working with myxobacteria, were stimulating and helpful.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Electron micrograph of a thin section of *Myxococcus xanthus* from a 48 hr. culture. Scale on print.

PLATE 2

Figs. 2-6; scale on prints.

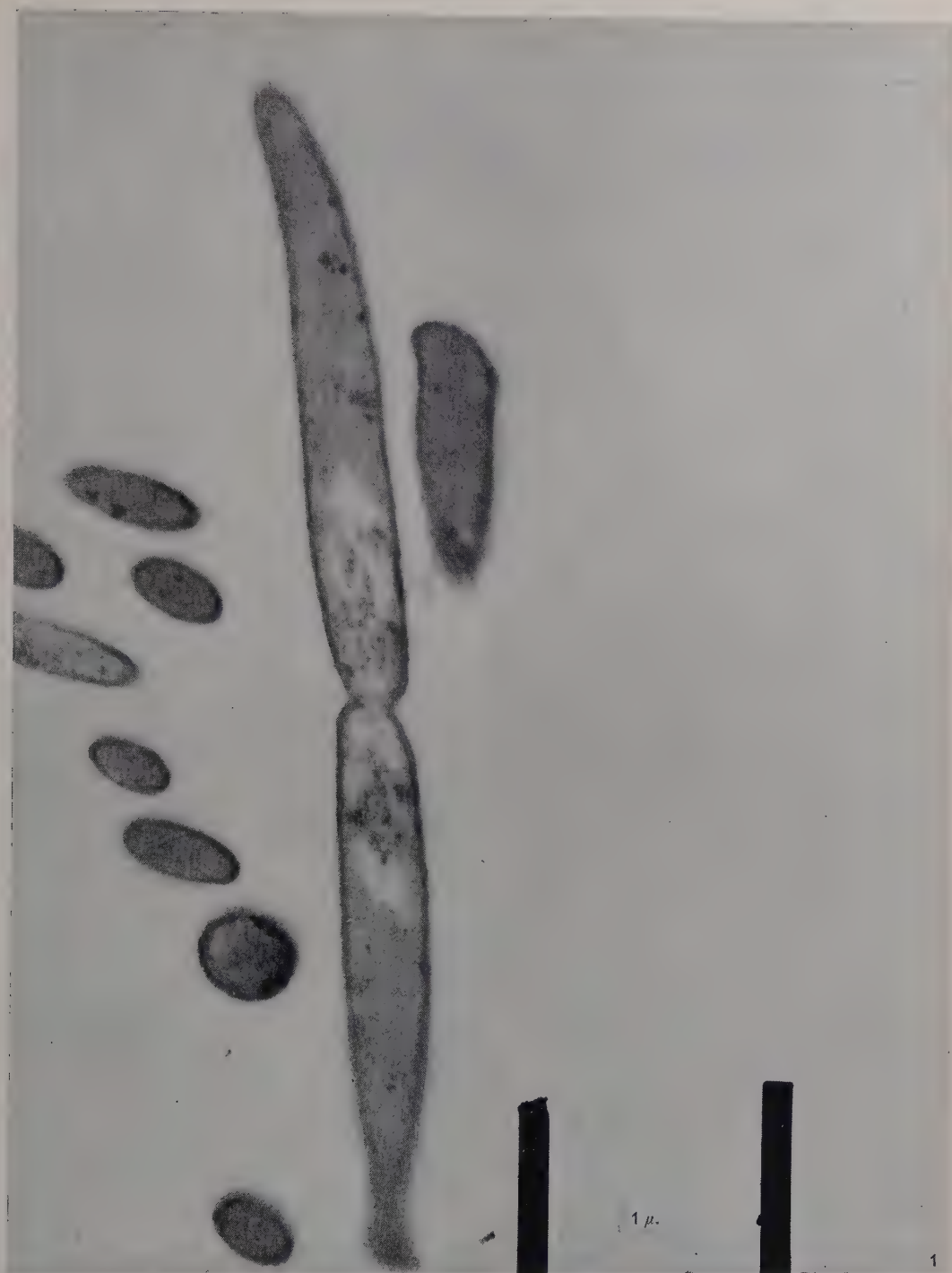
- Fig. 2. Phase-contrast microscopy of vegetative cells of *Myxococcus xanthus* from a 48 hr. culture.
- Fig. 3. Phase-contrast microscopy of *M. xanthus* (48 hr.) after cells have partially lysed at 45° in medium.
- Fig. 4. Phase-contrast microscopy of *M. xanthus* (48 hr.) after cells have completely lysed at 45° in medium.
- Fig. 5. Electron micrograph of a thin section of *M. xanthus* (48 hr.). Culture partially lysed in medium at 45°.
- Fig. 6. Electron micrograph of air dried *M. xanthus* (48 hr.). Culture partially lysed in medium at 45°. Chromium shadowed; negative print.

PLATE 3

Figs. 7-13; scales on prints.

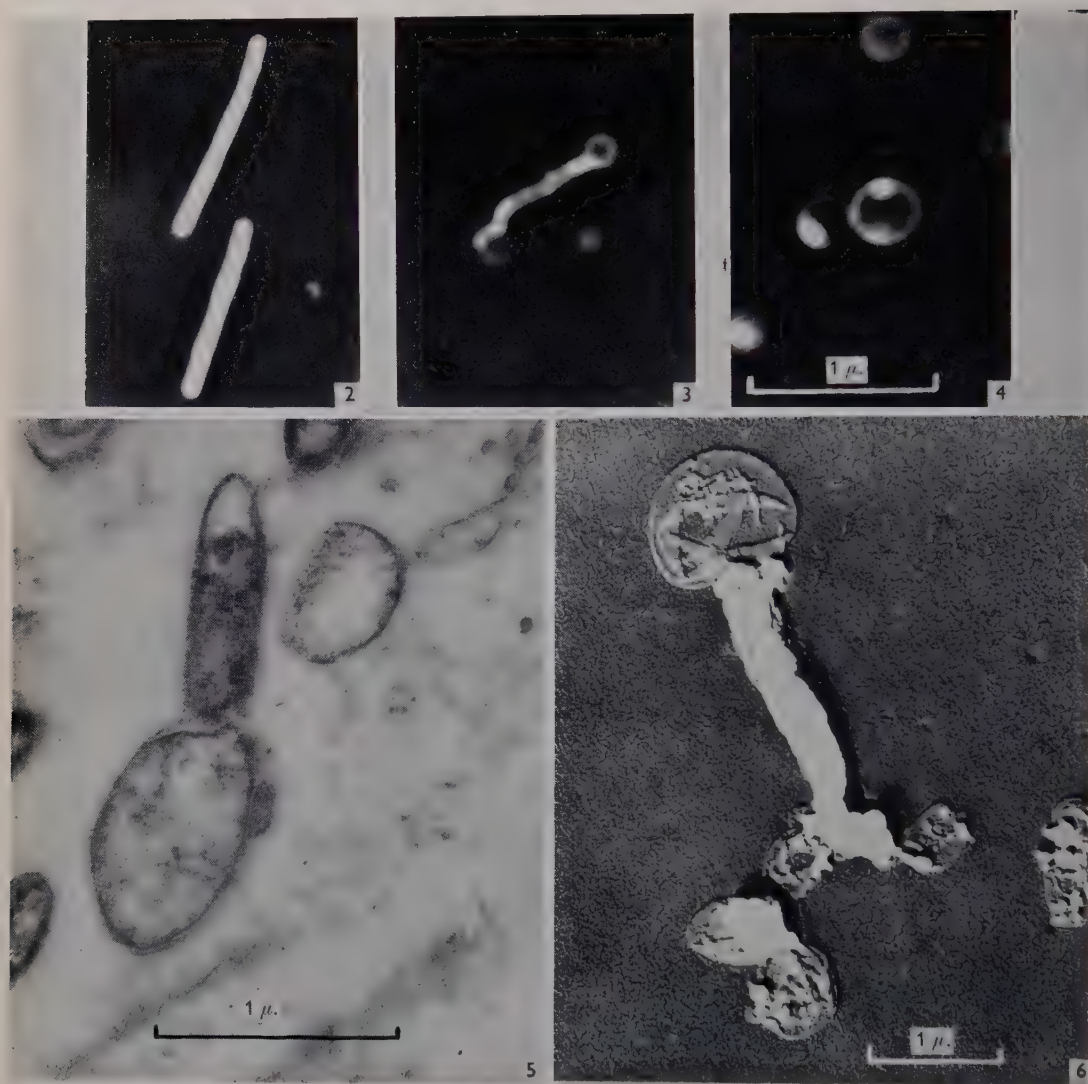
- Fig. 7. Electron micrograph of air-dried *Myxococcus xanthus* (48 hr.). Culture completely lysed in medium at 45°. Chromium shadowed; negative print.
- Fig. 8. Electron micrograph of thin sections of cells (48 hr.) lysed in medium at 45°.
- Figs. 9-11. Early stages of 'rounding-up' of *M. xanthus* (12 hr.). Cells in 0.5 M-sucrose at 30° for 30-90 min. Phase-contrast microscopy.
- Fig. 12. Round bodies of *M. xanthus* (12 hr.) in 0.5 M-sucrose at 30° after 120 min. Phase contrast.
- Fig. 13. Electron micrograph of a thin section of *M. xanthus* (12 hr.). Sections of cells 'rounded-up' in 0.5 M-sucrose at 30° after 120 min. Cells fixed in 2% (w/v) formaldehyde.

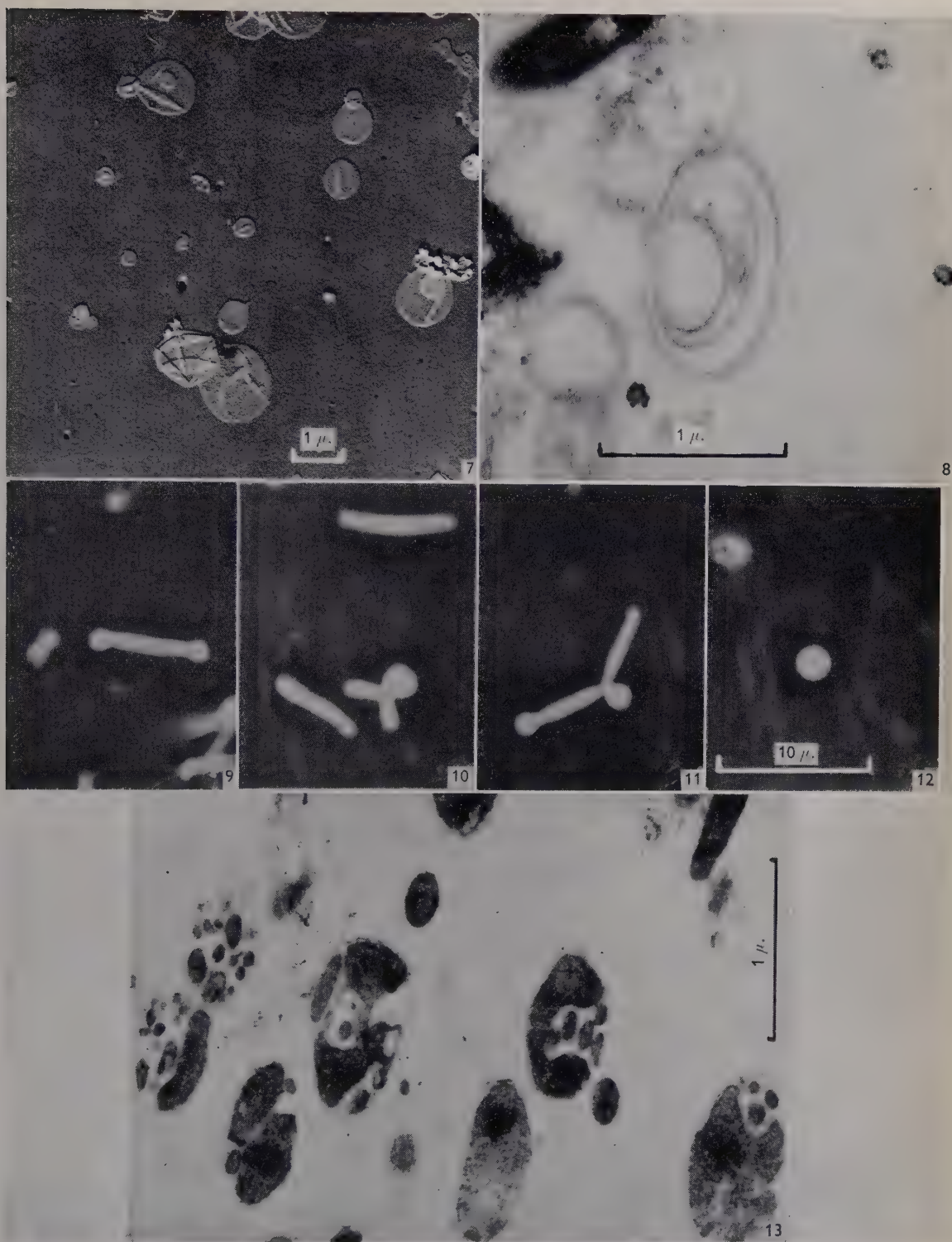
(Received 10 January 1958)



D. J. MASON & D. POWELSON—LYSIS OF MYXOCOCCI. PLATE 1

(Facing p. 70)





D. J. MASON & D. POWELSON—LYSIS OF MYXOCOCCI. PLATE 3

Genetic Recombination in *Vibrio cholerae*

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SUMMARY: Some pairs of genetically marked strains of *Vibrio cholerae* when grown together and plated under selective conditions give rise to greater number of colonies than either strain plated alone; in the case of other pairs in which this phenomenon did not occur, large number of presumptive recombinants were obtained when one of the strains was made lysogenic with a particular phage. Although the small number of markers made it impossible to do mapping studies, the evidence is strongly suggestive of recombination, probably phage-mediated transduction.

The literature on cholera contains many reports on the diverse variability of *Vibrio cholerae* which appeared to White (1937) to be without parallel in bacteriological lore. He examined some of these reports and made personal studies of some of the alleged variants, pointing out discrepancies which could not be explained. The only changes in this organism which were fully confirmed in later years were antigenic variations from Ogawa to Inaba and from smooth to rough forms (Shrivastava & White, 1947), which appeared to result from mutations (Bhaskaran & Gorrill, 1957). It seemed that genetic studies based on the techniques recently evolved for *Escherichia coli* and *Salmonella* were necessary to show how far the specific characters of *V. cholerae* may be altered. In this work genetical recombination is reported in *V. cholerae* and a possible mechanism suggested.

MATERIALS AND METHODS

Strains of Vibrio cholerae. A rough strain 129, requiring purine (Pu^-) for growth, was found to be lysogenic for a phage, also designated 129. For the sake of consistency with the terminology used in other strains this strain was designated 129 (129)⁺ to indicate the presence of phage 129. The following strains were found to be sensitive to phage 129: 6 (Inaba type, prototrophic), 20 (Inaba type, prototrophic), C₄ (Inaba type, prototrophic, non-motile), S^R (streptomycin-resistant), 23 (Ogawa type, prototrophic) 121 (Ogawa type, methionine-requiring (M^-)) and 121 S^R, a streptomycin-resistant derivative of 121. These strains could be made lysogenic with phage 129 and, for example, strain 20 after lysogenization would be designated 20 (129)⁺.

The antigenic type of these strains and the presumptive recombinants isolated in the experiments was determined by slide agglutination tests with suitably diluted mono-specific 'O' antisera (Ogawa, Inaba and Rough).

Media. *Nutrient broth* was used for the cultivation of *Vibrio cholerae*. It contained peptone (British Drug Houses Ltd., England), 0.5 % (w/v); Lab-Lemco (Oxo Laboratories, England), 0.1 % (w/v); sodium chloride (A.R.) 0.5 % (w/v); yeast extract (Difco Laboratories, Detroit), 0.1 % (w/v) in distilled

water, adjusted to pH 8 and sterilized in the autoclave at 15 lb./sq.in. for 15 min.

Nutrient agar. This was prepared by adding agar powder (British Drug Houses Ltd., England), 1.5% (w/v) to the nutrient broth.

Minimal agar. This was the minimal agar of Bhaskaran & Rowley (1956), containing ammonium sulphate and glucose as sole nitrogen, carbon and energy sources.

Technical procedures for recombination

Recombination for prototrophs. Ten ml. amounts of broth, distributed in 50 ml. conical flasks to provide a shallow layer of the medium, were seeded with 10^9 organisms of each strain individually or together. After 15 hr. incubation at 37° , the cultures were centrifuged at 2500 rev./min. for 15 min. and the deposits were washed with minimal medium. The final deposits were suspended in 1 ml. fluid minimal medium from which 0.1 ml. were seeded on minimal agar. Viable counts were made at the same time. After 72 hr. incubation at 37° , the numbers of prototrophic colonies which appeared from each culture and from the mixture were counted.

Recombination for streptomycin-resistant motile flares. Transduction of motility was studied in detail by Stocker, Zinder & Lederberg (1953). The method adopted for recombination in the present experiments was essentially the same as that for obtaining prototrophs but loopfuls of the centrifuged deposits were streaked on semi-solid gelatin agar (Edwards & Bruner, 1942) to which streptomycin had been added to a concentration of 500 μ g./ml.; after 24 hr. incubation at 37° the flares which arose from streptomycin resistant recombinants were counted.

RESULTS

Studies on lysogenic strains of *Vibrio cholerae* showed that strain 121 and its streptomycin-resistant mutant were reliable indicators for several lysogenic strains. When broth culture supernatants of the lysogenic cultures were spotted on a lawn of 0.4% (w/v) agar seeded with the indicator, according to the method of Adams (1950), turbid plaques with narrow clear lytic edges appeared after overnight incubation at 37° . It seemed possible to utilize these indicator strains to identify potential transducing phages among the lysogenic strains by direct crosses with them in the same manner in which genetic transduction was originally discovered in *Salmonella* (Zinder & Lederberg, 1952). With nutritional and other markers differentiating the lysogenic and indicator strains, selective methods would reveal if any genetic exchange occurred between the two.

One out of 26 such crosses proved to be of interest. In this the strains 129 (129)⁺ (purine exacting) and 121 S^B (methionine exacting) were crossed and it will be seen from Table 1 that the number of prototrophs isolated from the mixed culture was higher than in control single cultures and that they appear with frequency of 0.6 in 10^6 . They were lysogenic for phage 129, producing plaques on the parent strain 121 S^B.

These results suggest transfer of methionine independence from 129 (129)⁺ to 121 S^B, possibly mediated by phage 129. Strain 129 (129)⁺ was further crossed with the non-motile strain C₄ by the method described above and gave motile streptomycin-resistant flares and the fact that all the unselected markers were from one parent (Table 2) again suggested the transfer of one marker

Table 1. *Numbers of presumptive recombinants obtained in crosses between strains of Vibrio cholerae, using 10⁹ parent organisms*

Parent strains, and number of colonies produced by each, and by mixture, on minimal medium + streptomycin*				
Lysogenic (presumptive donor†) strains	No.	Non-lysogenic strain	No.	Mixed strains (cross), no.
129 (129) ⁺ (Pu-S ^B)	36†	121 S ^B (M-S ^B)	12†	600†
6 (prototroph S ^B)	1	121 S ^B (M-S ^B)	.	1
20 (prototroph S ^B)	0	121 S ^B (M-S ^B)	.	0
23 (prototroph S ^B)	0	121 S ^B (M-S ^B)	.	0
6 (129) ⁺ (prototroph S ^B)	0	121 S ^B (M-S ^B)	.	95
20 (129) ⁺ (prototroph S ^B)	0	121 S ^B (M-S ^B)	.	234
23 (129) ⁺ (prototroph S ^B)	0	121 S ^B (M-S ^B)	.	102

Parent strains, and number of 'flares' produced by each, and by mixture, in semi-solid agar + streptomycin*				
Lysogenic (presumptive donor†) strain	No.	Non-lysogenic strain	No.	Mixed strains (cross no.)
129 (129) ⁺ (motile Pu-S ^B)	0	C ₄ (prototroph non-motile S ^B)	0	+
6 (prototroph motile S ^B)	0	C ₄ (prototroph non-motile S ^B)	0	0
23 (prototroph motile S ^B)	0	C ₄ (prototroph non-motile S ^B)	.	0
6 (129) ⁺ (prototroph motile S ^B)	0	C ₄ (prototroph non-motile S ^B)	.	+
23 (129) ⁺ (prototroph motile S ^B)	0	C ₄ (prototroph non-motile S ^B)	.	+

* 500 µg. ml.

† On hypothesis of transduction.

++ indicates numerous confluent flares.

‡ No of colonies on minimal medium without streptomycin.

Table 2. *Details of unselected markers of isolated recombinants obtained in crosses between strains of Vibrio cholerae*

Lysogenic parent strain		Non-lysogenic parent strain		Medium used to select recombinants	Selected markers	No. examined and details of unselected markers of commonest class of recombinant	Other classes
Strain	Characters	Strain	Characters				
129 (129) ⁺	(Rough M+S ^B Pu ⁻)	121 S ^B	(Ogawa M-S ^B Pu ⁺)	Minimal medium	Pu ⁺ , M ⁺	200 Ogawa S ^B	None
6 (129) ⁺	Inaba S ^B)			Minimal medium + streptomycin	M ⁺ , S ^B	{ 94 Ogawa	1 Inaba
20 (129) ⁺	(Inaba S ^B)					{ 100 Ogawa	None
129 (129) ⁺	(Rough M+S ^B Pu ⁻)	C ₄	(Inaba non-motile S ^B)	Semisolid agar + streptomycin	Motility, S ^B	{ 20 Inaba proto-trophic	None
23 (129) ⁺	(Ogawa S ^B)					{ 20 Inaba	None

(motility) from 129 (129)⁺ to C₄. It may be remarked that C₄ is a stable non-motile strain and has not so far revealed the occurrence of motile mutants in several tests on semi-solid gelatin agar.

It was not possible to test the role of phage 129 as a transducing agent by means of cell-free lysates since a sufficiently high titre of phage could not be obtained. However, after lysogenizing one of the parent strains it has been

possible to demonstrate crossing in systems previously infertile. Such studies were carried out with strains 6, 20 and 23, none of which could be crossed with 121 S^R to yield streptomycin-resistant prototrophs or with C₄ to yield motile streptomycin-resistant flares; however, derivatives of strains 6, 20 and 23 made lysogenic with phage 129 gave positive results when crossed with 121 S^R or C₄ (Table 1). Here again the unselected markers of the recombinants isolated were those of the non-lysogenic strain (but in the case of the crosses 23 (129)⁺ × 121 S^R and 6 (129)⁺ × C₄ no unselected markers were available for examination). It will be seen from Table 2 that in the cross 6 (129)⁺ × 121 S^R, 94 out of 95 recombinant colonies tested had the unselected antigenic marker of the non-lysogenic strain, whereas in 20 (129)⁺ × 121 S^R, all the colonies tested were like the non-lysogenic strain in this respect. The lone exception was perhaps a streptomycin-resistant mutant of 6 (129)⁺. Isolates from recombination tests for motility gave similar results. It will be seen from Table 1 that the occurrence of streptomycin-resistant mutants in strains 6, 20 and 23 with an inoculum of 10⁹ organisms was quite rare which permitted the use of streptomycin containing media to isolate presumptive recombinants from crosses involving these strains.

DISCUSSION

The existence of genetic mechanisms in bacteria is now well established, although the number of species in which genetic interchanges have been shown to occur is still very limited and the importance of these phenomena in nature has still to be elucidated. The process of genetic exchange between bacterial strains may take place by one of several distinct mechanisms, e.g. transformation (Avery, McCleod & McCarty, 1944), conjugation (Tatum & Lederberg, 1947) and transduction (Zinder & Lederberg, 1952).

The experimental data presented and summarized in Table 1 implicate phage 129 in recombination in *Vibrio cholerae*, since the introduction of phage 129 as a lysogenic phage into a strain can render it fertile. By analogy with *Salmonella* (Zinder & Lederberg, 1952) it can be suggested that recombination takes place by transduction and that phage 129 is the transducing agent. This hypothesis is supported by the lysogenic state of the recombinants, at least in the cross 129 (129)⁺ × 121 S^R, and by the fact that the unselected markers are those of the non-lysogenic strain (Table 2).

However, the evidence, though very strong, is circumstantial and until cell- and DNA-free lysates of phage 129 can be used to transduce characters into sensitive cells the hypothesis of phage-mediated transduction cannot be considered proven.

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The Infra-Red Absorption Spectra of Lactobacilli

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SUMMARY: The infra-red absorption spectra of 76 strains of lactobacilli representing 11 different species were examined. With only a few exceptions, the spectra could be grouped into 5 distinct spectral types, each corresponding to a *Lactobacillus* species or a group of related species. From a study of acetic acid extracts, it has been found possible to relate spectral differences to the relative concentrations of nucleic acids and carboxylate groups in the different organisms.

Since the original work of Stevenson & Bolduan (1952) a number of reports have been published on the application of infra-red spectroscopy to the differentiation of bacteria and other micro-organisms. A paper by O'Connor, McCall & Du Pré (1957) gives references to most of the relevant work published in the U.S.A., and to this should be added the papers by Thomas & Greenstreet (1954), Lembke & Kaufmann (1954), Greenstreet & Norris (1957) and Rideal & Adams (1957).

The classification of the lactobacilli is based on the work of Orla Jensen (1919, 1943) who defined three groups of lactobacilli which he further subdivided into a number of species, and also on that of Pederson (1938) who further defined the heterofermentative group. Much recent work has been done on the classification of these organisms; studies on the biochemical characteristics (Rogosa, Wiseman & Mitchell, 1953; Briggs, 1953*a*; Wheeler, 1955*a, b*; Davis 1955; de Man, 1956*a, b*) and serological characteristics (Sharpe, 1955*a*; Sharpe & Wheeler, 1957) have made possible the precise identification of many species of lactobacilli. Some of this work has now been confirmed by chromatographic analysis (Mattick, Cheeseman, Berridge & Bottazzi, 1956; Cheeseman, Berridge, Mattick & Bottazzi, 1957) and it was thought that the infra-red absorption spectra might provide useful information about the relationships between these organisms. The present work describes attempts to classify a number of species of *Lactobacillus* by this method, using representative strains from a collection of lactobacilli at this Institute; these have previously been examined here and identified by some or all of the techniques mentioned above.

For the purpose of studying species classification by spectroscopic methods, it was thought preferable to examine the spectra of whole organisms rather than the spectra of extracts as used by O'Connor *et al.* (1957). The method of sample preparation, which has been in use now for several years in our laboratory, consisted of incorporating the freeze-dried organisms in potassium bromide disks. The preparation of such disks has been described by Ford & Wilkinson (1954) and has also been applied successfully to the study of bacteria by Rideal & Adams (1957).

METHODS

Strains of lactobacilli

The organisms used for this work consisted of 76 strains of lactobacilli from our collection, representing 11 different species. They included laboratory strains obtained from many different sources, and a number of fresh isolates from Cheddar cheese. The organisms examined were: 7 strains of *Lactobacillus acidophilus*, 6 of *L. helveticus*, 7 of *L. bulgaricus*, 6 of *L. lactis*, 2 of *L. delbrueckii*, 22 of *L. casei* (including 9 strains belonging to the serological group C of Sharpe & Wheeler, 1957; 10 strains of serological group B; 3 strains which gave the characteristic biochemical reactions of *L. casei* but did not fall into any serological group), 2 strains of *L. casei* var. *rhamnosus*, 8 strains of *L. plantarum*, 6 of *L. fermenti* and 10 of *L. brevis* group. The *L. brevis* group comprised low-temperature heterofermentative lactobacilli falling into Briggs's (1953a) group VI; these could be further subdivided into 6 strains of *L. brevis*, 3 of *L. buchneri* and 1 of *L. viridescens*, a new species described by Niven & Evans (1957).

Cultures were grown in a modification of Briggs's (1953b) tomato juice medium consisting of: glucose 2%; Evans's peptone 1.5%; NaCl 0.5%; Yeastrel 0.3%; Tween 80 0.01% (all w/v); and tomato juice 10% (v/v), the final pH value being 6.6. Strains of *Lactobacillus acidophilus*, *L. bulgaricus*, *L. helveticus*, *L. lactis*, *L. delbrueckii* and *L. fermenti* were incubated at 37° for 24 hr.; and strains of *L. casei*, *L. plantarum* and *L. brevis* were incubated at 30° for 48 hr. All organisms were subcultured at least three times in 10 ml. tomato juice medium before being examined. For preparing samples for spectroscopic studies, organisms were grown in 20 ml. tomato juice medium for the times specified above, centrifuged, and the organisms washed three times in sterile distilled water, the final washing taking place in the ampoules in which the organisms were to be freeze-dried. As lactobacilli are non-pathogenic, no attempt was made to kill the cultures before further treatment.

The washed organisms were then freeze-dried for about 6 hr. at a final vacuum of 0.01 mm. Hg in an Edwards's LC 5 Freeze-drier, and no differences were observed in the spectra of the same cultures which had been dried for 3, 6 or 12 hr. About 1 mg. samples of the freeze-dried powder were ground and mixed intimately with 200 mg. of dried potassium bromide (Analar) in a vibratory ball mill, and the material was then pressed under vacuum to provide optically clear disks $\frac{1}{2}$ in. in diameter. A second disk prepared from potassium bromide was placed in the comparison beam of the spectrophotometer. The instrument used was a Grubb Parsons S3A double-beam spectrophotometer fitted with a sodium chloride prism, recording at a speed of 1 μ /min. In all cases spectra were recorded over the 2–15 μ wavelength range, but since the 5–13 μ region includes the bands of major interest, this limited region only is illustrated in the figures.

For this type of work, involving the observation of small changes in the shape and contour of absorption bands, it was found necessary to ensure that all specimens were examined under identical instrumental conditions. Duplicate

spectra of many of the organisms were examined on different occasions, in order to check the reproducibility of the method. Owing to slight variable mechanical losses in the preparation of the sample disks, it was not possible to record duplicate spectra at exactly the same intensity, but in most cases the actual differences in transmission were only of the order of a few % and the different spectra could be compared directly on a roughly quantitative basis.

When organisms were not thoroughly washed, traces of tomato juice and other constituents of the medium remained on them (Sharpe, 1955*b*). Under such conditions the freeze-dried samples were hygroscopic and in air were rapidly converted into sticky masses which were impossible to manipulate.

Liquid medium was used in preference to agar-solidified medium for growing the bacteria as it is much more convenient to use for lactobacilli and gave quite satisfactory results. To achieve maximum growth on agar plates, lactobacilli should be incubated under anaerobic conditions whereas in liquid medium this is not necessary.

Greenstreet & Norris (1957) mentioned that the addition of carbohydrate to a medium may result in large changes in the spectra of bacteria. Since carbohydrate is necessary for the growth of lactobacilli, it must be added to media for growing them. As the present study is concerned only with the genus *Lactobacillus*, it was possible to use the same medium throughout, so that any effects due to the carbohydrate should be the same throughout.

RESULTS

General characteristics

From a detailed study of the spectra of the 76 strains so far examined, it has been found possible to group the spectra into 5 distinct types, each corresponding to a *Lactobacillus* species or group of related species. With a few exceptions, all the strains examined could readily be classified into the 5 types according to certain prominent features in their spectra. In the first instance, the spectra can be divided into 2 distinct types X and Y. Type X is characterized by a relatively strong band near 960 cm.^{-1} and by the presence of an intense band near 1230 cm.^{-1} . In most cases, the 1320 cm.^{-1} band is as strong as, or stronger than, the broad 1060 cm.^{-1} band. Broad weak bands are usually observed in the $910\text{--}770\text{ cm.}^{-1}$ region and strong shoulders are not usually observed on the higher frequency side of the 1060 cm.^{-1} band. Type Y is characterized by the weakness or complete absence of the 960 cm.^{-1} band, coupled with the high intensity of the 1060 cm.^{-1} band compared with that of the 1230 cm.^{-1} band. The $910\text{--}770\text{ cm.}^{-1}$ region is usually free from weak bands and the 1060 cm.^{-1} band usually carries a number of weak shoulders, particularly on the higher frequency side.

Within the X and Y spectral types, it is possible to subdivide the spectra further into two types L or M. Most of the strains belonging to each species are of the same L or M type. Type L is characterized by the relatively low intensity of the 1400 cm.^{-1} band, as compared with the 1450 cm.^{-1} band. In

addition to being broader in shape, the 1400 cm.^{-1} band is usually almost equal in intensity to the 1450 cm.^{-1} band. Type M is characterized by a relatively strong 1400 cm.^{-1} band compared with the 1450 cm.^{-1} band. The absorption in the trough between the 1650 cm.^{-1} and 1550 cm.^{-1} bands in the M type is also greater than in the L type.

A third feature is the weak band at 1725 cm.^{-1} which is sometimes observed on the side of the strong 1650 cm.^{-1} band. This weak band is only discernible as a shoulder and is observed in the spectra of *Lactobacillus plantarum* and some of the *L. brevis* organisms. The strains classified according to these spectral features correspond to the various species and also to the larger groups of Orla Jensen (1919), as shown in Table 1.

Table 1. Comparison of spectral types and *Lactobacillus* species

Spectral type	<i>Lactobacillus</i> species	Orla Jensen group	General physiological characters of group
XL	<i>L. acidophilus</i>	Thermobacterium	Homofermentative, usually growing at high temperatures and unable to grow at low temperatures
	<i>L. lactis</i>		
	<i>L. delbrueckii</i>		
XM	<i>L. bulgaricus</i>		
	<i>L. helveticus</i>		
YLa*	<i>L. plantarum</i>	Streptobacterium	Homofermentative, growing at low temperatures
YLB†	<i>L. casei</i>		
YM	<i>L. brevis</i> group	Betabacterium	Heterofermentative, growing at low temperatures
	<i>L. fermenti</i>	Betabacterium	Heterofermentative, usually growing at high temperatures and unable to grow at low temperatures

* 1725 cm.^{-1} band present. † 1725 cm.^{-1} band absent.

Figures 1 and 2 show spectra of strains of each species. The spectra of the strains within each group will be discussed in detail below.

Spectra of strains in each serological group

Lactobacillus acidophilus. Spectral type XL, example A1, Fig. 1. Strains examined: A1, A3, A4, A12, A15, N4, BF3. The spectra of A3 and BF3 were atypical in that both spectra showed only a weak band at 960 cm.^{-1} , in addition to a relatively weak 1230 cm.^{-1} band. Such features are more characteristic of Y spectral type organisms. However, both strains possessed the typical biochemical characteristics of *L. acidophilus*.

Lactobacillus lactis. Spectral type XL, example L3, Fig. 1. Strains examined: L1, L3, L4, L18, L19, AH7. No exceptions were found in the spectra so far examined, and within this group it was most difficult to distinguish spectra of individual strains.

Lactobacillus delbrueckii. Spectral type XL, example D2, Fig. 1. Strains examined: D2, D10. Since only two strains were examined in this group, it would be improper to assign a definite spectral type for the whole group. Strains D2 and D10 appeared to show XL spectral-type features, although the 960 cm.^{-1} band in the spectrum of strain D10 was not very strong.

Lactobacillus helveticus. Spectral type XM, example H21, Fig. 1. Strains examined: H20, H21, ME10, MP8, AH4, PR3. All the strains in this group showed X type spectral features and although strains H20, H21, ME10 and MP8 showed M type features, strains AH4 and PR3 showed an intermediate intensity for the 1400 cm^{-1} band and could not definitely be classified according to the L or M spectral types.

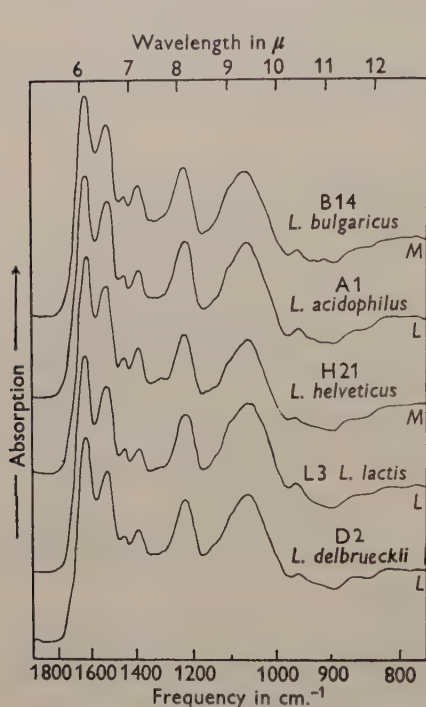


Fig. 1

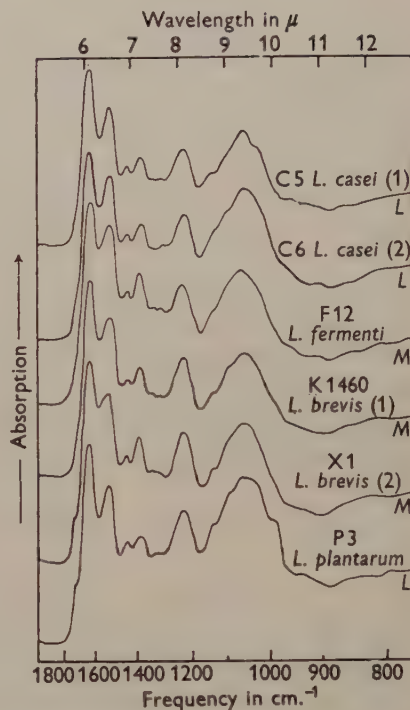


Fig. 2

Fig. 1. Spectra of type X organisms. (L and M indicate spectra of type L or M.)

Fig. 2. Spectra of type Y organisms. (L and M indicate spectra of type L or M.)

Lactobacillus bulgaricus. Spectral type XM, example B14, (Fig. 1), Strains examined: B2, B12, B14, B15, B18, B21, B24. Within this species, all the strains gave spectra with pronounced X type characteristics. Most spectra were of the M type, but B21 and B24 showed L type features. Both of these strains gave the characteristic biochemical reactions of *L. bulgaricus*, but B24 did not show the typical serological reactions of this species.

Lactobacillus casei. Spectral type YL, examples C5 and C6 (Fig. 2). All the strains within this group showed clearly Y type spectral features and, in addition, most spectra were of the L type, although the L features were not always very pronounced. The intensity of a shoulder near 1030 cm^{-1} on the side of the 1060 cm^{-1} band enabled the *L. casei* strains to be split into two subgroups, purely on the basis of their spectra. In subgroup 1 the 1030 cm^{-1} shoulder stood out against the general slope of the band, but in subgroup 2,

no shoulder was visible. The strains classified into the two subgroups according to their spectra were:

Subgroup 1: A10, B163, B142, C2, C5, C10, C15, C18, CP3, H2, L16, CW193, MR3A.

Subgroup 2: C6, C9, C24, P1, P9, P11, O7, A121, A148, D3, SM4.

Those corresponded closely to the subdivision of the species *Lactobacillus casei* into two serological groups (Sharpe, 1955*a*; Sharpe & Wheeler, 1957), one group B, containing strains of *L. casei* and the other group C, also containing strains of *L. casei* and, in addition, of *L. casei* var. *rhamnosus*. The biochemical characteristics of the *L. casei* strains in both serological groups were the same and strains could only be distinguished by serological tests. The strains falling into spectral subgroup 1 all belong to serological group C, with the exception of L16 and CP3 which are serological group B, and the strains falling into spectral subgroup 2 all belong to serological group B, with the exception of strains P9, D3 and A148. These last three have not yet been placed in serological group B, but they show serological relationships to other members of this group, which suggests that they are also members of it. This is confirmed by their spectra. P9 shows a weak band at 960 cm^{-1} , together with a strong 1230 cm^{-1} band, these features being suggestive of an X type organism.

Within subgroup 1, H2 and C10 could be differentiated from the other organisms within the subgroup by the presence of a weak shoulder near 1105 cm^{-1} . These two were strains of *L. casei* var. *rhamnosus*, a variety which can be distinguished from *L. casei* biochemically (Rogosa *et al.* 1953) and also serologically by the possession of a particular type antigen (Orland, 1950; Sharpe, 1955*a*). Within subgroup 2, C6, P1, and P11 were all of the same serological type and could not be differentiated separately on the basis of their spectral features.

Lactobacillus plantarum. Spectral type YL, example P3, Fig. 2. Strains examined: P3, P5, P12, P14, AR1, AB41, H268, SS140. The spectrum of H268 was completely different from that of any other *lactobacillus* so far examined in that the structure of the broad 1060 cm^{-1} band was very complex. The spectra of both this strain and of P12 showed a strong band near 960 cm^{-1} similar to the X type organisms. H268 appears to be a characteristic strain of *L. plantarum*, but P12, however, is atypical biochemically, although it reacts with the *plantarum* group serum.

Apart from these two cases, all the spectra were of type Y and, in addition, the shoulder near the 1725 cm^{-1} was observed. This latter feature was found to be peculiar to all the *L. plantarum* spectra and to a few of the *L. brevis* spectra. Most of the strains in this group showed L type feature, but P5, P12 and SS140 showed M type features.

Lactobacillus brevis group. Spectral type YM, examples K1460 and X1, (Fig. 2). Strains examined: K1460, BC1, D210, V7, T3, T6, X1, X6, SL6, S41A. Apart from strain S41A, which is not a typical *L. brevis* biochemically or serologically, and has recently been assigned to the new species *L. viridescens* (Niven & Evans, 1957), all the spectra in this group showed both Y

type and M type characteristics. The intensity of the weak shoulder near 1725 cm.^{-1} enabled most of the *L. brevis* spectra to be divided into two subgroups according to whether this band was absent (1) or weak (2). X6 showed the band of intermediate intensity, but the remaining strains could easily be subgrouped as follows:

Type 1: K1460, BC1, D210, V7, T3.

Type 2: X1, S41A, T6, SL6.

There appears to be no biochemical or serological evidence at present for such a further division of this group; the three strains of *L. buchneri* BC1, D210 and T3, all fall into type 1, but so do 2 strains of *L. brevis*.

Lactobacillus fermenti. Spectral type YM, example F12 (Fig. 2). Organisms examined: F1, F3, F4, F12, F15, 010. All the strains in this group showed pronounced Y type and M type features in their spectra. In most cases, the 1400 cm.^{-1} band was stronger than the 1230 cm.^{-1} band.

Origin of bands in spectra

The problem of accounting for the observed absorption bands in terms of molecular structure is particularly difficult, since in almost every case a number of different chemical entities give rise to absorption at the same frequency. Rideal & Adams (1957) have been able to identify the components responsible for some of the bands by studying the spectra of various extracts from the whole organisms and the cell-wall material. The spectra of a number of the extracts used for the paper chromatographic technique (Mattick *et al.* 1956) have been examined in the course of this work and these have assisted in the interpretation of the spectra of the whole organisms.

Figure 3 shows the spectrum of the organism BC1 (*L. brevis* group) examined (a) as whole organisms, and (b) as the residue after extracting with 10% acetic acid at 37° for 2 hr., followed by washing three times with water. Figure 3(c) shows the spectrum of the combined extracting and washing liquids, the water and acetic acid having been removed by warming *in vacuo* for several days. The spectra (a) and (b) were obtained from samples which had been freeze-dried and made up in potassium bromide disks. Spectrum (c) was obtained from a sample which had been cast as an aqueous film heat dried on a barium fluoride plate. Owing to absorption by the barium fluoride, the latter spectrum could only be examined up to about 13μ .

The spectrum of whole organisms (a) is of the type YM as shown by strains of the *L. brevis* group but the spectrum of the residue after extraction (b) appears to have many characteristics of the X and L spectral types.

The ionized carboxyl group ($-\text{CO}_2^-$) is known to show a pair of absorption bands near 1600 and 1400 cm.^{-1} (Bellamy, 1954). From an examination of the spectra of Fig. 3, it can be seen that the acetic acid treatment removes components absorbing at both these frequencies. Since amino acids absorb also near 1600 cm.^{-1} (Bellamy, 1954) and these are known to be removed by the acetic acid treatment, a study of the 1400 cm.^{-1} band gives more precise in-

formation about the carboxyl group. Methyl groups ($-\text{CH}_3$) absorb near 1400 and 1450 cm^{-1} (Bellamy, 1954), so that some of the loss of intensity of the 1400 cm^{-1} band may well be due to the removal of smaller molecules (e.g. amino acids) containing a relatively large number of methyl groups. An examination of the exact frequencies of this band shows that it occurs at 1397 , 1380 and 1412 cm^{-1} in (a), (b) and (c) respectively. Since the methyl group usually absorbs at frequencies slightly less than 1400 cm^{-1} and the carboxyl group at slightly higher frequencies, it is considered that the main effect of the acetic acid extraction is to remove materials containing ionized carboxyl groups rather than methyl groups.

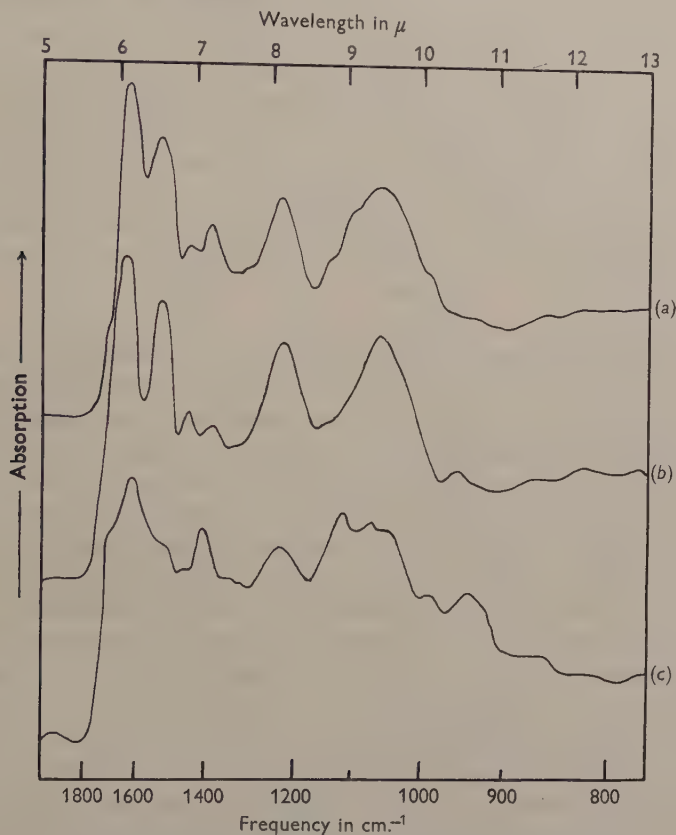


Fig. 3. Spectra of BC1 (*L. brevis* group) and extracts: (a) whole cells, (b) residue after acetic and acid extraction (c) material extracted.

A study of the intensity of the 1400 cm^{-1} band showed that this was most intense in the species *Lactobacillus fermenti* and *L. brevis* (subgroup 2), suggesting that such organisms contained a relatively high carboxylate ion concentration. It is of interest to note that paper chromatography of the acetic acid extracts of organisms in these groups showed particularly intense ninhydrin spots corresponding to the positions of glutamic and aspartic acids, i.e. acidic amino acids.

A study of the shoulder near 1725 cm.^{-1} suggests that this band may be due to un-ionized carboxylic acid groups ($-\text{CO}_2\text{H}$) or O-acetyl groups rather than ester carboxyl groups occurring in fatty materials. This band is removed by acetic acid treatment and appears in the extracting solvent. Although this change is not very apparent in Fig. 3, it was much more obvious in the spectra of T 6 (*Lactobacillus brevis* group) and its extracts. Repeated grinding of several strains with carbon tetrachloride failed to remove this band from the spectra.

Levine, Stevenson, Bordner & Edwards (1955) have shown that the spectra of polysaccharides isolated from bacteria show very strong absorption near 1060 cm.^{-1} , and also weaker absorption near 1230 cm.^{-1} , the former being associated with the hydroxyl groups. Sutherland & Tsuboi (1957) have shown that nucleic acids also absorb in the same two regions of the spectrum, and their published spectra show that the relative intensities of the two bands are almost the same. In addition, the nucleic acid spectra show a band near 960 cm.^{-1} which does not appear in the spectra of the polysaccharides. This band can therefore be used as a measure of the nucleic acid content. Since it appears more intense in the X-type lactobacilli, it seems probable that such strains contain relatively more nucleic acid than the Y-type strains. Further confirmation is provided by the higher intensity of the 1230 cm.^{-1} band in the spectra of the X-type organisms.

Extraction with acetic acid leaves a residue with an apparently higher nucleic acid content (Fig. 3*b*). This change appears to be due to the removal of other constituents which absorb at 993 and 943 cm.^{-1} on either side of the 960 cm.^{-1} band. The shape and intensity of the 1060 cm.^{-1} band in the spectrum of the extract (Fig. 3*c*) suggests that some polysaccharide material has also been extracted, whilst the 1725 cm.^{-1} band may be due partly to carboxylic acid or O-acetyl groups of the polysaccharide. It therefore seems likely that the extraction technique employed here removes both amino acids and polysaccharides, leaving a residue with increased nucleic acid content.

Greenstreet & Norris (1957) have shown that the spectra of some aerobic spore-forming bacilli show very pronounced changes during the growth period. When a number of typical lactobacilli were examined at intervals during their growth period, only small changes were observed in the spectra. X-type strains appeared to show very little change, although during the early growth stages of some of the Y-type strains, the 960 cm.^{-1} band appeared almost as intense as in the spectra of the X-type organisms. This presumably indicated a higher nucleic acid concentration during the early stages of growth.

In view of these observations, it might at first seem that the division of the organisms into X and Y types arises from differences in chemical composition brought about by using two different sets of growth conditions. In this work the organisms were usually grown under the conditions which ensured maximum growth. A selection of strains normally grown at 37° for 24 hr. were however grown at 30° for 48 hr. and vice versa. As expected, slight changes in spectra were observed, but only in a few cases did the different growth conditions cause changes large enough to change the X or Y character of the spectrum. In none of the cases examined was it possible to change the L or

M character of the spectrum. Further evidence in this matter is provided by the *Lactobacillus fermenti* and *L. brevis* strains, which all gave rise to YM type spectra, although grown for 24 hr. at 37° and 48 hr. at 30° respectively.

DISCUSSION

The classification of lactobacilli according to spectral type corresponds in many respects to other methods of classification. Orla Jensen's (1919) broad division of the lactobacilli into *Thermobacteria*, *Streptobacteria* and *Betabacteria*, which was based on their physiological and nutritive characteristics, confirms some of our groupings; strains possessing the X type of spectra fall into the *Thermobacteria*, whilst strains possessing the Y type fall into the *Streptobacteria* or the *Betabacteria*. This division is also associated with the manganese requirements of the organisms. It was observed by Orla Jensen (1943) and later confirmed by one of us (M.E.S.) that, whilst the *Thermobacteria* require only traces of manganese for optimum growth, the *Streptobacteria* and *Betabacteria* require relatively large amounts. Further subdivision of the X and Y groups into the L and M types by their spectra also corresponds to other methods of classification. The X group—the *Thermobacteria*—are divided into the XL type containing *Lactobacillus acidophilus* and *L. lactis*, which are similar biochemically, and also the less closely related *L. delbrueckii*, and the XM type containing *L. bulgaricus* and *L. helveticus*, which both belong to the same serological group and also possess very similar biochemical characteristics. The Y group also shows further subdivisions, corresponding to the *Streptobacteria* (type YL) which are further divided into species *L. casei* and *L. plantarum*, and the *Betabacteria* (type YM) which are not further divided into species.

From this study of the spectra of the lactobacilli and their extracts, it would seem that organisms with the X-type spectra contain relatively larger amounts of nucleic acid than those of the Y type. The M-type organisms appear to contain relatively larger amounts of ionized carboxylate groups than the L type organisms, although the evidence is insufficient to decide whether such groups are due largely to the polysaccharide or amino acid components.

Thus it can be seen that the classification of lactobacilli according to their infra-red spectra is related to changes in the chemical composition of the organisms and is in agreement with the differentiation of species by other methods.

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The Cell Walls of *Platymonas*

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SUMMARY: Electron micrographs of cell walls of the unicellular green flagellate *Platymonas subcordiformis* revealed no evidence of a fibrous construction. A preparation of walls was isolated, hydrolysed, and analysed by paper chromatography. The major components indicated were galactose and a uronic acid; glucose was not detected. It was concluded that the cell walls of this alga are not composed of cellulose.

A strong case can be made for the close phylogenetic relationship between higher plants and green algae. Among the accepted features of resemblance are the nature of the plastid pigments, the storage of starch, and the presence of a cellulose wall. However, there are few cases in which the chemical nature of the walls of green algae has been critically examined. Most of the evidence is indirect, being based on their birefringence, solubility in cuprammonium solutions, or staining reactions (Fritsch, 1935). The walls of many of the larger filamentous forms have been shown under the electron microscope to be composed of fibrils which in size agree with those of cellulose from Angiosperms; X-ray diffraction studies indicate that they are, in fact, some form of cellulose (Nicolai & Preston, 1952). In *Spirogyra*, half of the cell-wall material can be dissolved by successive extractions with hot dilute alkali and acid, and yields galactose and xylose on hydrolysis; but the remainder appears to consist of cellulose (Kreger, 1957). In some species of unicellular green algae, however, cellulose may not be present at all. The walls of several members of the Chlorococcales, including *Ankistrodesmus falcatus* (Sponsler & Bath, 1950), *Chlorella variegata*, *C. protothecoides*, *Coccomyxa dispar*, *Scenedesmus obliquus*, *S. quadricauda* and *Selenastrum minutum* (R. A. Lewin, personal observations), appear completely smooth in electron micrographs. Those of two species of Chlamydomonas are striate (Lewin, Owen & Melnick, 1951), but, whereas the width of the bands (233 Å) is in close agreement with that characteristic of cellulose fibrils, their regular parallel arrangement is not. Among other members of the Volvocales, the cell walls of *Haematococcus* (Motte, 1953) and of *Platymonas* (Kylin, 1935; Margalef, 1946) do not exhibit typical staining reactions for cellulose. Bailey & Neish (1954) analysed the cell-wall residues of *Chlorella* cells from which the contents had been removed by exhaustive digestion with acetic acid and sodium chlorite. Of the insoluble 'holocellulose' 96% proved to consist of a polyglucose hemicellulose, soluble in hot aqueous KOH. True cellulose, if present, can therefore constitute only a small fraction of the wall substance of *Chlorella*.

In many unicellular algae, including members of the Volvocales and the Chlorococcales, young cells are formed endogenously and liberated by rupture of the parental wall. This permits the separation of shed walls from cells

without prior chemical treatment, by the purely mechanical method of differential centrifugation. In this way the cell walls of *Platymonas* can readily be obtained free from other cellular material.

METHODS

Bacteria-free strains of *Platymonas subcordiformis* Hazen were isolated from tidal pools near: New Haven, Connecticut; Woods Hole, Massachusetts; La Jolla, California; and Halifax, Nova Scotia. Cultures were grown in sea-water media enriched with nitrate, phosphate and trace elements (Lewin, 1954), under constant light (2500 lux) and temperature (20°). Turbulence and a supply of carbon dioxide were maintained by the continuous passage of a stream of sterile air. Methods employed for the detection of bound sulphate, sugars, and amino acids in hydrolysates of the cell walls are described elsewhere (Lewin, 1956, 1958; Partridge, 1948).

RESULTS

Microscopic examination of suspensions of all isolates revealed an abundance of thin rod-like bodies among the organisms. These could be distinguished from shed flagella by their greater width and rigidity, but their nature remained obscure until examined under the electron microscope. It was then apparent that these rod-like structures were cast-off cell walls, more or less rolled up into scrolls (Pl. 1, fig. 2-5). Since, after each binary division, the liberation of daughter cells leaves one empty parental wall, cells and shed walls might be expected to occur in equal numbers. Thin sections of cells fixed with osmium tetroxide showed the wall as a smooth, homogeneous, electron-dense layer, perforated only to permit the exit of the flagella (Pl. 1, fig. 6).

The Woods Hole organism was grown under auxenic conditions for 6 weeks. The supernatant medium was then separated by centrifugation at 150-350g for 30 min. and discarded. The residue was re-suspended in filtered sea water, and was centrifuged in a tapered 15 ml. tube at 20-70g for 15 min. Most of the cells were thereby precipitated, leaving a milky suspension containing the cell walls. These were separated from the supernatant suspension by centrifugation at 500-700g for 15 min., and were then washed several times by suspension in distilled water and re-centrifugation. A pure white fraction was obtained, consisting of cell walls virtually free from whole cells. After drying at room temperature in a vacuum desiccator over concentrated sulphuric acid, the yields from a 1.5 l. culture were: cells, 1900 mg.; cell walls, 26.4 mg.

The dried cell-wall preparation readily flaked off the surface of the glass container as a colourless, transparent film. A fragment was soaked in water and examined under the microscope; the cell walls appeared unchanged. Another portion was examined under crossed polaroids; although it was folded in various ways, there was no indication of birefringence such as was shown by a strand of cotton cellulose. An X-ray diffraction diagram of this material was kindly prepared and examined by Mr C. K. Johnson (Massa-

chusetts Institute of Technology); it showed no characteristic spacings, indicating that the material was quite amorphous. A micro-Kjeldahl determination, carried out on a 5 mg. sample, indicated the presence of 0.55% nitrogen. Another 5 mg. sample was examined for organically-bound sulphate; this was found not to exceed 1% and was considered to be negligible.

Finally, hydrolysates of this wall preparation were chromatographed on paper, together with known sugars, etc., for reference. Chromatograms were sprayed with aniline phthalate for hexoses, pentoses and uronic acids; with dimethylaminobenzaldehyde for hexosamines; and with ninhydrin for amino acids. The predominant components appeared to be galactose and a uronic acid, together with traces of arabinose. Glucose and hexosamine were not detected. Traces indicating several amino acids (alanine, arginine, aspartic acid, glutamic acid, glycine, leucine/iso-leucine, methionine/valine, proline, serine, threonine) appeared on the chromatograms, suggesting that the small amount of organic nitrogen might be attributed to protein contamination. A similar preparation of cell walls (23 mg.) from the Halifax strain gave on hydrolysis essentially the same results, though in this case there was also a faint indication of mannose.

DISCUSSION

Paper chromatographic analysis of cell-wall hydrolysates, together with other evidence from electron microscopy, an X-ray diffraction diagram, etc., supports previous indications from staining reactions that the walls of *Platymonas subcordiformis* are not composed of cellulose. The question may be raised whether *P. subcordiformis* is a true green alga, since it is the only member of the Chlorophyceae which has been found to possess flagella of the 'tinsel' type (Pitelka & Schooley, 1955. Dr Pitelka has kindly confirmed this fact for all of the four strains we isolated.) A critical analysis of the plastid pigments might be expected to throw further light on this matter.

The electron micrographs were prepared in the School of Medicine, Yale University, and, with the assistance of Mr D. E. Philpott, in the Marine Biological Laboratory, Woods Hole, Mass.

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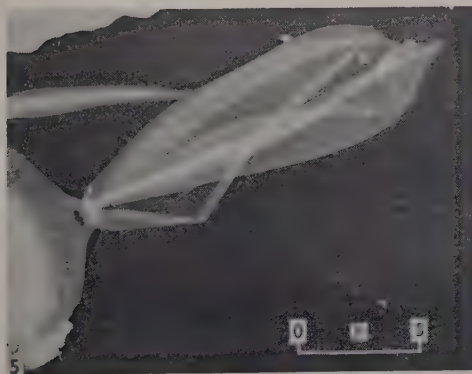
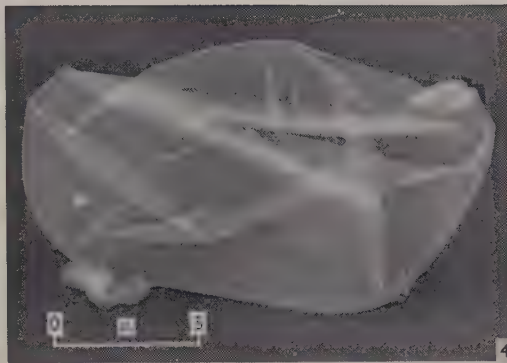
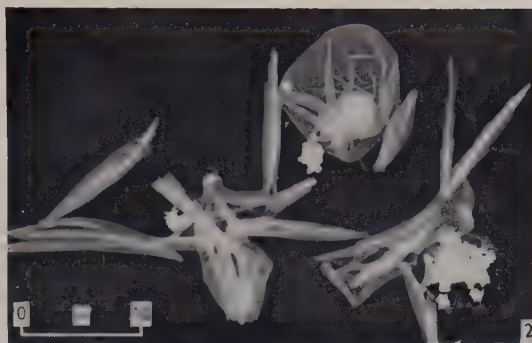
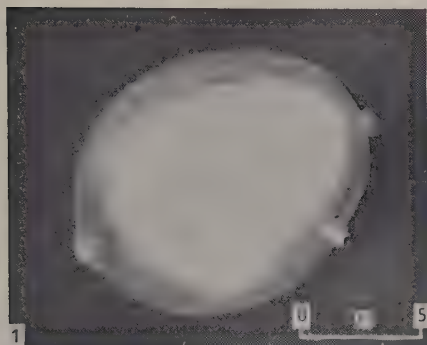
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EXPLANATION OF PLATE 1

- Fig. 1. *Platymonas subcordiformis*. One organism, showing contents drawn away from wall.
- Figs. 2-5. Cell walls, showing various stages in rolling up to form scrolls.
- Fig. 6. Section, showing portion of a cell and three more or less rolled-up cell walls.

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Production *in vitro* of the Toxin of *Bacillus anthracis* Previously Recognized *in vivo*

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SUMMARY: A lethal oedema-producing toxin has been made *in vitro* in serum cultures of *Bacillus anthracis* which is identical with the toxin originally recognized *in vivo*. Toxin is produced by the attenuated immunogenic Sterne strain as well as by the virulent N.P. strain. Previous non-recognition of the toxin in cultures was due to its early appearance and rapid disappearance under ordinary growth conditions. A toxin-destroying mechanism was demonstrable in organisms from cultures grown for more than a few hours. Attempts to maintain the toxin concentration or to increase it by addition of various nutritives to batch culture failed; only by continuous culture have the organisms been kept in their toxigenic growth phase. Traces of the toxin appear in cultures in tryptic meat broth; these are increased by the addition of large molecular constituents of serum which appear to play an important role in toxin production.

Until recently the product of *Bacillus anthracis* which is responsible for death in anthrax was unknown. No lethal endo- or exotoxin had been found in cultures of the organism *in vitro* (Eurich & Hewlett, 1930; Sobernheim, 1931; King & Stein, 1950). As a result of working with organisms grown *in vivo* rather than with those from artificial culture, Smith, Keppie & Stanley (1955) showed that *B. anthracis* does form a specific lethal oedema-producing exotoxin. It was found in the plasma of guinea-pigs dying of anthrax. Although the characteristics of the toxin were clearly established it was not demonstrated in cultures *in vitro* until some time had elapsed. The failures to detect the toxin under these conditions was due to its exceptionally early appearance followed by its rapid disappearance, which meant that toxin was only detectable during a very short period, at an unusually early stage of the culture. The present paper describes the production of the toxin *in vitro*, also its properties and attempts to improve its yield and to prolong its activity under various culture conditions.

METHODS

Organisms used. The virulent N.P. strain of *Bacillus anthracis* and the attenuated Sterne and H.M. strains were used.

Guinea pig blood and serum. Blood was removed from guinea pigs by cardiac puncture and collected into heparin (10 i.u./ml.) or immediately defibrinated, under as aseptic conditions as possible. After removing the blood cells, the plasma contained a small amount of haemoglobin and was used for toxin production within 2 days of its collection.

Anthrax toxin produced in guinea pigs. This was obtained as described by Smith *et al.* (1955).

Albumin I (Armour). This was Fraction V of bovine plasma prepared by the Cohn & Edsall separation. It was not especially purified and formed a brown solution.

Albumin II. This was a special sample of bovine albumin which showed no evidence of heterogeneity when examined electrophoretically or in the ultracentrifuge.

γ -Globulin. This was Fraction II of bovine plasma prepared by the Cohn & Edsall separation, and supplied by L. Light and Co.

Guinea-pig haemoglobin. Well washed guinea-pig red blood corpuscles were haemolysed with distilled water; after centrifugation the solution was freeze dried.

Bovine and sheep haemoglobin. Prepared as described for guinea-pig haemoglobin.

Estimation of bacterial growth. The number of bacterial chains (2–8 bacilli)/ml. of culture was obtained from the average number of chains/microscope field of a standard film (Keppie, Smith & Harris-Smith, 1955); 1 chain/field was equivalent to 1.5×10^7 chains/ml. ($\pm 50\%$). In media consisting of mainly tryptic meat digest, *Bacillus anthracis* grew in longer chains than in plasma. For this reason an estimate of bacterial growth was obtained from the extinction of light by the turbid solutions, measured in an EEL spectrophotometer. Appropriate blanks were used in these estimations, and in Table 6 the readings of the EEL spectrophotometer are quoted as measures of bacterial growth.

Assay of toxin in culture filtrates. The skin test for oedema production described by Smith *et al.* (1955) was used for routine assay of samples of culture which had been filtered sterile through Millipore membranes. The average result of duplicate assays are recorded here in the manner described by these authors, i.e. 8:40 indicates a lesion of skin-fold thickness 8 mm. and diameter 40 mm. Tests for lethality in mice and guinea pigs were carried out as described by Smith *et al.* (1955).

RESULTS

Production of toxin from the virulent N.P. strain of Bacillus anthracis

The initial experiments were related to the fact that toxin appeared in the blood of guinea pigs dying of anthrax when the bacterial count was *c.* 1×10^7 chains/ml. and the animal was *c.* 7 hr. from death (Smith *et al.* 1955). In preliminary experiments, heparinized or defibrinated guinea-pig blood (9 ml.) was inoculated with a suspension (1 ml., 3×10^8 spores) of *Bacillus anthracis* strain N.P. spores in tryptic meat digest, which had been incubated at 37° for 30 min. to germinate the spores. The mixtures were shaken at 37° in a Warburg bath at 120 strokes/min. with air containing 20% (v/v) CO₂ continuously passing over them. Samples tested by the skin-assay showed that the toxin concentration was maximal at 4 hr. (size of skin lesion 8:35–10:40) and had disappeared by 7–8 hr. after the start of inoculation.

Further experiments soon established that serum could be used as effectively as whole blood; also that the maximum concentration of toxin and its subsequent rapid disappearance were not greatly affected by varying the following

conditions of cultivation: the presence or absence of heparin; rate of shaking or aeration; temperatures of 28°, 34° or 37°; different batches of guinea-pig blood or serum. In all cases, as soon as a certain amount of growth had occurred (count *c.* $5-10 \times 10^7$ fully developed chains/ml.) the toxin concentration was maximal and then rapidly disappeared (at counts greater than $1-2 \times 10^8$ chains/ml.). The size of the initial inoculum was somewhat critical if a high maximal concentration of toxin was to be obtained. The results in the upper part of Table 1 show that an inoculum size lower than the optimal resulted in a slightly less rapid appearance and disappearance of the toxin, but the maximal toxin concentration was not as high as that obtained with the optimal size of inoculum. A large inoculum resulted in a very rapid appearance and disappearance of the toxin. The optimal inoculum size and the conditions of culture described in Table 1 were used for the production of toxin for all subsequent work.

*Production of toxin by the virulent non-capsulated immunogenic
Sterne strain of Bacillus anthracis*

The data in the lower part of Table 1 show that toxin was produced by the Sterne strain of *Bacillus anthracis*. The pattern of early appearance and rapid disappearance of toxin and the existence of an optimal inoculum size for maximal toxin production was the same as for the virulent strain. The capsulated attenuated H.M. strain of *B. anthracis* did not produce demonstrable toxin when tested under the conditions in Table 1.

Toxin concentrations similar to those quoted in Table 1 were obtained when the N.P. or the Sterne strains were grown for short periods in human, calf or sheep serum under the conditions described in Table 1.

*A comparison of the properties of the anthrax toxin prepared in vitro
and in vivo*

The toxins formed *in vitro* by the N.P. and Sterne strains of *Bacillus anthracis* were compared with that produced by the N.P. strain *in vivo*. The results shown in Table 1 demonstrate the difficulty of obtaining cultures at the peak of toxin production. For the following tests, cultures were taken at 15-30 min. intervals between 3.5 and 4.5 hr. after the start of incubation. Only in this way could toxin be obtained which approached in potency that invariably present in the plasma of guinea pigs dying of anthrax. When, in addition to the optimal conditions outlined in Table 1, cultures were grown in an atmosphere of 20% (v/v) CO₂ in air, the filtrates contained on the average slightly more toxin than those grown in air without added CO₂. The use of CO₂ appeared to some extent to broaden duration of the toxin peak, thus making it easier to obtain samples of maximum toxicity. Carbon dioxide + air was used whenever filtrates of maximum toxicity were required. On average, the Sterne strain of *B. anthracis* produced slightly higher concentrations of toxin than did the N.P. strain.

Oedema production. In the standard skin test for oedema production lesions of 7-10:35-40 were obtained after injecting toxic filtrates from N.P. and

Table 1. *The rapid appearance and disappearance of the anthrax toxin in cultures of virulent (N.P.) and avirulent (Sterne) strains of Bacillus anthracis: the effect of size of inoculum*

Culture conditions: fresh slightly haemolysed, defibrinated guinea pig serum (15 ml.) to which the inoculum is added as a suspension in tryptic meat digest (3.3 ml.) which had been incubated at 37° for ½ hr. to germinate the spores. Cultures were shaken at 37° in 250 ml. flasks with cotton wool plugs at 100 strokes/min.

Inoculum (no. germinated spores/ml.) N.P. strain (virulent)	Age of culture (hr.)										
	0	2	2.5	3	3.5	4	4.5	5	6	8	10
3×10^8	Toxin* Count† (30)	Nil	5:27	7:36	6:34	6:30	5:19 (23)	Nil	Nil	—	—
6×10^7 (i.e. optimal)	Toxin Count (4.5)	Nil	6:20 (4.5)	—	6:24	8:38	8:37 (9)	6:37	Trace	Nil	Nil
8×10^7	Toxin Count (3)	Nil	Trace	—	—	6:27	6:28 (4.5)	6:35	7:34 (12)	Trace (24)	Nil
1×10^7	Toxin Count (1)	Nil	Trace	—	—	—	5:17 (1.5)	—	6:20 (3)	6:20	Nil (11)
Sterne strain (avirulent)											
1.5×10^8	Toxin Count (15)	Nil	6:35	8:40	8:40	7:39	6:36 (15)	4:25	Nil	Nil	Nil
3×10^7 (i.e. optimal)	Toxin Count (3)	Nil	6:28 (3)	—	7:32	8:38	9:42 (7)	6:27	Nil	Nil	Nil
0.75×10^7	Toxin Count (0.75)	Nil	5:16	—	5:18	—	7:30 (1.5)	—	8:35 (3)	5:22 (6)	Nil (15)

* Size of skin lesion (Smith *et al.* 1955): 5:27 = lesion of skin-fold thickness 5 mm. and lesion diameter 27 mm.
† No. of chains $\times 10^7$ /ml. of culture.

The almost equal counts/ml. of bacterial chains at 0 and 2 hr. are somewhat misleading; there was an increase of bacterial protoplasm during this period from the small germinated spore to the mature chain of *B. anthracis* containing 2-8 organisms.

Sterne strains of *Bacillus anthracis*. The lesions formed by toxin produced *in vivo* were 8-10:40-45. The null point (Smith *et al.* 1956) of the toxin prepared *in vivo* was at a dilution of 1/256; the corresponding dilutions for toxic filtrates from the Sterne and N.P. strains were 1/256 and 1/128, respectively.

Lethality to mice and guinea pigs. Results of lethality tests on toxic filtrates from subcultures of N.P. and Sterne strains of *Bacillus anthracis* are shown in Table 2.

Table 2. *The lethality of Bacillus anthracis toxic filtrates for mice and guinea pigs and its specific neutralization*

Antiserum (i) was prepared by hyperimmunizing horses with living avirulent *B. anthracis* spores; 1/5 of volume of toxic filtrate was added.

Antiserum (ii) was prepared by hyperimmunizing horses with the immunizing antigen of Belton & Strange (1954); 1/5 of volume of toxic filtrate was added.

Toxic filtrates came from cultures in air containing 20% (v/v) CO₂.

All injections made intravenously, and for guinea pigs, freeze-dried material was used at double the original strength.

Sample	Skin lesion 0.2 ml.	Mice* (deaths/no. injected)			Guinea pigs* (deaths/ no. injected)	
		1.5 ml.	1 ml.	0.5 ml.	20 ml.	10 ml.
Toxin from N.P. strain						
Alone†	7:38	37/46	24/41	0/10	7/9	1/3
With antiserum (i)	Nil.	0/5	—	—	0/2	—
With antiserum (ii)	Nil	0/5	—	—	—	—
Toxin from Sterne strain						
Alone†	8:40	24/29	57/73	11/15	9/9	9/15
With antiserum (i)	Nil	0/14	0/24	—	0/5	—
With antiserum (ii)	Nil	0/10	0/10	—	—	—

* For comparison, the approx. L.D.₅₀ of the anthrax toxin produced *in vivo* for the mouse and the guinea pig was 0.4 ml. and 6 ml. respectively (Smith *et al.* 1955).

† When normal horse serum (1/5 volume of toxic filtrate) was mixed with the toxic filtrates it did not affect either lethality or oedema production.

Specific neutralization by antiserum. The anthrax toxin produced *in vivo* was specifically neutralized by the serum of horses hyperimmunized against anthrax by using live avirulent *Bacillus anthracis* spores (Sterne strain) or a non-toxic immunizing antigen produced *in vitro* by our colleagues Messrs Belton & Strange (1954). Similarly, the oedema production and lethality of toxic filtrates from cultures of N.P. and Sterne strains of *B. anthracis* were also neutralized by these two types of antiserum (see Table 2.)

Specific precipitation in Ouchterlony plates. Toxin produced *in vivo* formed a maximum of three precipitation lines when examined in a standard Ouchterlony diffusion system against either of the two sera described above (Stanley & Smith, personal communication). When compared on the same Ouchterlony plate with the toxin formed *in vivo*, toxin filtrates from cultures of N.P. and Sterne strains of *Bacillus anthracis* when concentrated fivefold, formed identical lines against both types of antiserum.

Presence of at least two components forming a synergic mixture. The anthrax toxin produced *in vivo* could be separated by ultracentrifugation into two

components—Factor I (deposit) and Factor II (supernatant fluid) (Smith *et al.*, 1956). When injected alone, Factor I had a much lower activity than the original toxin and Factor II (when free from Factor I) was devoid of activity; mixed together, they formed a fully toxic mixture. Our colleague Mr J. L. Stanley ultracentrifuged the toxic culture filtrates from both strains of *Bacillus anthracis* as described by Smith *et al.* 1956; after one ultracentrifugation for 16 hr. the unwashed deposit (Factor I) was separated from the supernatant fluid (Factor II approx. 8 ml.) and dissolved in saline (3 ml.). The results of skin tests for synergic action between these two factors and with the respective components of the toxin prepared *in vivo* are shown in Table 3. It is clear that Factors I and II are present in the toxin prepared *in vitro*.

Table 3. *Synergic action of Factors I and II from the toxic filtrates of Bacillus anthracis produced in vitro with each other and with Factors I and II from toxin produced in vivo.*

Strain of <i>Bacillus anthracis</i> from which toxic filtrate prepared	Skin tests for toxin				
	Ultracentrifugal fractions injected alone				
	Unwashed supernatant deposit		Synergic mixtures of Factor I (0.1 ml.) + Factor II (0.1 ml.)		
	Factor I* (0.1 ml.)	Factor II (0.1 ml.)	<i>in vitro</i> I <i>in vitro</i> II	<i>in vitro</i> I <i>in vivo</i> II	<i>in vivo</i> I <i>in vitro</i> II
<i>In vitro</i>					
Strain N.P.	8:24	Nil	11:30	9:40	10:40
Strain Sterne	9:35	Nil	11:40	11:38	10:35
<i>In vivo</i> †					
Strain N.P.	9:32	Nil	—	—	—

* These crude preparations of Factor I inevitably contain some Factor II which accounts for the relatively high inherent activity. Factor II can be freed from I by ultracentrifugation.

† For method of preparation see Smith *et al.* 1956.

Production of active immunity. Anthrax toxin prepared *in vivo* will actively immunize guinea pigs against subsequent infection with *Bacillus anthracis*. A 1/8 or 1/16 dilution of it will protect *c.* 50% of the animals in the test for immunizing activity described by Smith & Gallop (1956). A similar degree of protection in this test was obtained from 1/4 and 1/16 dilutions of toxic filtrates from 4 to 4½ hr. cultures of the N.P. and Sterne strains, respectively. After 7 hr. growth, when the culture filtrates were no longer toxic, they could still immunize although less effectively. Thus, an undiluted filtrate from a late culture of N.P. strain and a 1/2 dilution of a similar filtrate from Sterne strain protected 50% of the animals. This partial deterioration parallels the behaviour of the *in vivo* toxin when treated with various chemical reagents; it rapidly loses its toxicity but retains some ability to immunize.

Selective inhibition of toxin synthesis by 2-thiouracil. Tempest & Smith (1957) showed that *in vivo* 2-thiouracil interfered with toxin production by *Bacillus*

anthracis without significantly affecting its rate of growth. This antimetabolite had a similar action on toxin production by both N.P. and Sterne strains *in vitro* (Table 4).

Table 4. *The effect of 2 thiouracil on toxin synthesis in vitro by Bacillus anthracis*

Medium and conditions (optimal inoculum) of incubation as described in Table 1. The number of bacterial chains/ml. in the growing culture at the different times was not significantly different in control and experimental samples.

Medium	Organism	Duration of incubation		
		3.5 hr	4.5 hr.	6 hr.
		Skin reaction		
Control	Sterne	6:37	7:42	Nil
+thiouracil (0.1 %)	Sterne	4:21	4:20	tr
Control	N.P.	6:32	6:35	Nil
+thiouracil (0.1 %)	N.P.	5:30	5:27	Nil

The toxin-destroying action of Bacillus anthracis

The effect of bacterial population on toxin production. The results in Table 1 show that toxin production and destruction were related to the number of bacteria in the culture. As soon as a critical amount of growth had occurred (c. $5-10 \times 10^7$ fully developed chains/ml.), the amount of toxin was maximal and at greater amounts of growth it rapidly disappeared. This was confirmed by the following experiment. When non-toxic, 6-7 hr. cultures of the N.P. or Sterne strains of *Bacillus anthracis* were centrifuged and the deposited organisms resuspended in the same volume of medium (1 vol. tryptic meat broth + 9 vol. plasma), no toxin appeared on incubating this relatively high concentration of organisms. On the other hand, toxin was produced when the medium was inoculated with these organisms, when the inoculum was decreased to that normally used for toxin production. Thus, after 2-2.5 hr. of incubation, the toxin gave skin tests of 7-9:35-40. The toxin soon disappeared on further incubation, as the number of bacteria increased.

The toxin-destroying mechanism in organisms from cultures aged 7 hr. or more. It seemed likely that organisms found in the older cultures of *Bacillus anthracis* (N.P. and Sterne strains) could destroy their own toxin. Extracts of organisms from 2.5, 7 and 12 hr. cultures and the non-toxic filtrates from 7 and 12 hr. cultures were tested for their ability to destroy toxin when incubated with it (see Table 5). In contrast to toxigenic organisms from 2.5 hr. cultures, organisms from 7 and 12 hr. cultures contained a toxin-destroying mechanism which appeared to be essentially intracellular; at 7 hr. it was not present in the culture medium to any appreciable extent, but by 12 hr. it was clearly demonstrable, probably due to lysis of some organisms. This apparent change in the organisms between 2.5 and 7 hr. was supported by a difference in their infrared spectra; these examinations were made by our colleague Dr K. Norris to whom we are indebted.

Table 5. *The toxin-destroying mechanism in organisms from late samples of cultures of Bacillus anthracis (strains N.P. and Sterne)*

Mixture	Skin lesions produced by the mixtures (0.4 ml.) after 2 hr. incubation at 37°	
	Strain N.P.	Sterne strains
Sterile toxin* (1 vol.) mixed with 1 vol of:		
Saline	7:33	7:32
Streptomycin solution (2000 u./ml.)	7:35	7:33
Extract† of organisms from 2.5 hr. culture‡	7:29	7:31
Non-toxic filtrate from 7 hr. culture	7:35	6:31
Extract of organisms from 7 hr. culture	Nil	Nil
Non toxic filtrate from 12 hr. culture	Nil	Nil
Extract of organisms from 12 hr. culture	Nil	Nil
(a) Soluble (filtered sterile)	Nil	Nil
(c) Insoluble	Nil	Nil

* Toxic filtrates from 3½–4 hr. cultures under conditions as in Table 1.

† Bacterial extracts were prepared by shaking in a Mickle shaker with ballotini beads for ½–¾ hr. No organisms could be cultured from the disintegrates but streptomycin (2000 units per ml.) was added as a precaution since the turbid whole extracts could not be filtered sterile. The concentration of organisms used was equal (on the basis of turbidity) to the concentration in a normal 7 hr. culture prepared under the conditions described in Table 1.

‡ Conditions of culture as described in Table 1.

Attempts to increase yields of toxin and prolong its life in cultures

Quasi-continuous culture. Cultures (24 ml.) of both the N.P. and Sterne strains of *Bacillus anthracis* were incubated for 4 hr. at 37° under the optimal conditions described in Table 1, i.e. until toxin production was near its peak. Half (12 ml.) of the culture was then removed for assay and the medium replenished over the next hour by the addition, at 10 min. intervals, of six 2 ml. portions of warm medium. After 5 hr. of incubation, half (12 ml.) the culture was again removed and the same mode of addition of fresh medium repeated. The whole process was continued until the 8th hr. of incubation. The bacterial count of all samples of both strains did not depart significantly from 10⁷ bacterial chains/ml. At 4, 5, 6, 7 and 8 hr. after the start of incubation the sizes of the skin lesions in the assay for toxin were 6:37, 6:36, 6:37, 6:44 and 6:42 for the N.P. culture and 7:42, 7:40, 7:41, 7:46 and 8:47 for the Sterne culture. A comparison of these results with those given in Table 1 shows that when fresh medium was continually added and the bacterial count retained approximately the same by occasional removal of culture, then the high content of toxin which normally disappeared quickly in these cultures was maintained.

Batch culture. Apart from the application of this quasi-continuous culture system, we were not able to maintain peak toxicity or significantly to increase toxin concentration in the standard type of culture where the bacterial population was continually increasing. Attempts in this direction are described below.

(1) *Added nutrients.* The following four mixtures of amino acids, purine/pyrimidines and vitamins produced no significant difference from the control culture when added either alone (at the concentrations given in brackets and at 1/10, 1/50 and 1/500 of these concentrations) or when mixed together.

A. Glycine (20 mM), DL-alanine (2 mM), DL-valine (0.4 mM), DL-leucine (1 mM), DL-isoleucine (1 mM), DL-proline (1 mM), hydroxyproline (1 mM) DL-serine (2 mM), DL-threonine (0.4 mM), DL-norleucine (1 mM).

B. L-cystine (0.4 mM), DL-methionine (0.4 mM), DL-aspartic acid (1 mM), DL-glutamic acid (2 mM), DL-lysine (1 mM), L-arginine (1 mM), L-histidine (0.4 mM), DL-phenylalanine (1 mM), DL-tyrosine (1 mM), DL-tryptophane (0.2 mM).

C. Thiamin (2 μ M), riboflavine (2 μ M), nicotinamide (10 μ M), Ca pantothenate (2 μ M), pyridoxine (4 μ M) choline chloride (40 μ M), inositol (200 μ M), biotin (2 μ M) pimelic acid (200 μ M), folic acid (1 μ M) *p*-aminobenzoic acid (10 μ M).

D. Adenine (100 μ M), guanine (100 μ M), xanthine (100 μ M), hypoxanthine (100 μ M), cytosine (100 μ M), thymine (100 μ M), uracil (100 μ M), glutamine (1000 μ M), haemin (80 μ M).

The work of Tempest & Smith (1957), on the metabolic background to the production of the anthrax toxin *in vivo*, suggested that addition of coenzyme I (0.0025 %), coenzyme II (0.0025 %), uracil (0.1%), or nicotinamide (0.1%) might increase toxin production. The addition of nicotinamide and uracil did slightly increase toxin production but this increase was hardly significant.

(2) *Constant pH value.* The anthrax toxin is sensitive to changes in pH value (Smith, *et al.* 1955). The pH value of cultures did not fall outside the range 7.4–7.8 during 8 hr., and no change in the toxin production and destruction pattern was obtained by adding sodium phosphate to maintain a constant pH value. The addition of sodium bicarbonate (0.5 and 0.1 %) retarded the growth rate somewhat so that toxin was maintained for a slightly longer period.

(3) *Oxidizing and reducing substances.* Mr J. L. Stanley and Dr H. Smith (personal communication) have found that Factor 1 of the anthrax toxin is sensitive to oxidation and reduction. Addition of cystine (0.1 and 0.01 %), cysteine (1, 0.1 and 0.01 %) and KIO₃ (0.1 %) with a view to counteracting any oxidizing or reducing action of the organism, produced no significant change from normal. Anaerobic culture resulted in some toxin production of the same pattern as that in normal culture, but possibly slower.

(4) *Miscellaneous.* Addition of egg-yolk broth (1/5 vol.), arachis oil containing cholesterol (1/5 vol.), soya-bean meal containing trypsin inhibitor (0.5 %), and charcoal (0.1 %) produced no significant change from the normal pattern.

The influence on toxin production of high molecular weight components of serum

The experiments described above gave no clue as to the function of serum and tryptic meat digest medium in producing the right nutritional conditions for toxin production. The effectiveness of the added mixtures of smaller

molecular nutritives suggested that it was the larger molecular components of serum which were important in toxin production. The following experiments support this view and also indicate the part played by tryptic meat broth.

When serum alone was used in the standard procedure, toxin production and disappearance was delayed but the maximal content was not significantly lower than when the normal mixture of serum and 10 % tryptic meat digest was used; the function of tryptic meat digest seemed to be to accelerate growth (see top of Table 6). Tryptic meat digest in the standard medium can be replaced by its ultrafiltrate without affecting toxin production or growth (see Table 6), showing that it is the small molecular components which are important. On the other hand, toxin was not produced on a medium of tryptic meat digest or its ultrafiltrate + the ultrafiltrate of serum, although normal growth of the organism occurred. Hence the large molecular weight components of serum appeared to be most important in toxin production.

Production of small amounts of toxin in tryptic meat broth; the influence of serum and other material of high molecular weight

Only the Sterne strain of *Bacillus anthracis* regularly produced a small amount of toxin in tryptic meat digest (see Table 6). The N.P. strain produced a similar quantity only in certain batches of tryptic meat digest. A significant increase in toxin production was brought about by the addition of serum (20 %, v/v; see Table 6), but the amount of toxin was not equal to that in the normal mixture of serum and 10 % (v/v) tryptic meat digest even when the proportion of serum was increased to 50 %.

The nature of the high molecular weight components in serum which may be of importance is not clear. Addition of a sample of albumin which had not been specially purified, significantly increased toxin production but a pure sample of albumin and a sample of γ -globulin did not. The impure sample of albumin was brown and its absorption at 410 m μ indicated the presence of haemoglobin. The addition of guinea-pig haemoglobin (not specially purified) increased toxin production but no more than the impure sample of albumin or serum. A fivefold decrease of the haemoglobin concentration did not significantly change the effect. Horse and bovine haemoglobin acted similarly, but haemoglobin could not be replaced by haematin or iron salts.

At no time in this work with media composed mainly of tryptic meat digest, did the concentration of toxin reach that obtained during growth in the conventional mixture of serum with 10 % (v/v) of tryptic meat broth. The rather low concentrations of toxin obtained were however maintained in the presence of far higher bacterial populations than in the conventional mixture (see Table 6).

DISCUSSION

The toxin responsible for death from anthrax has now been produced *in vitro* from a virulent strain and from an attenuated immunogenic strain of *Bacillus anthracis* growing in a mixture of serum + tryptic meat digest. The toxin appears identical with that found *in vivo*, and cultures of the Sterne strain have a

Table 6. *Toxin production in mixtures of tryptic meat broth with guinea pig serum and other materials of high molecular weight*

Medium		Skin tests for toxin and turbidity* measurements of bacterial growth at 37° by EEL Spectrophotometer (in brackets)															
Tryptic meat digest % v/v.	Other constituent of mixture	N.P. Strain								Sterne strain							
		Age of culture (hr.)								Age of culture (hr.)							
		3	4	5	6	7	8	3	4	5	6	7	8	3	4	5	6
10%	Serum	7:35 (3)	7:35 (5)	tr (7)	6:30 (2)	Nil (22)	—	6:35 (3)	7:40 (6)	6:30 (7)	tr (11)	Nil (19)	—	6:35 (3)	7:40 (6)	6:30 (7)	tr (11)
Nil	Serum	5:20	5:25 (1)	7:32 (2)	6:30 (2)	6:31 (3)	—	tr (4)	6:32 (1)	8:35 (1.5)	6:29 (2)	tr. (7)	—	tr (4)	6:32 (1)	8:35 (1.5)	6:29 (2)
10% ultrafiltrate	Serum	7:30 (4.5)	6:36 (5)	5:23 (7)	Nil (12)	—	—	6:37 (5)	7:44 (5)	7:35 (5)	tr. (9)	—	—	6:37 (5)	7:44 (5)	7:35 (5)	tr. (9)
10%	Serum ultrafiltrate	Nil (3)	Nil (7)	Nil (13)	Nil (12)	—	—	—	Nil (7)	Nil (16)	Nil (14)	—	—	—	Nil (7)	Nil (16)	Nil (14)
10% ultrafiltrate	Serum ultrafiltrate	Nil (2)	Nil (2)	Nil (5)	Nil (8)	—	—	—	Nil (6)	Nil (10)	Nil (11)	—	—	—	Nil (6)	Nil (10)	Nil (11)
80%	Saline	Nil (13)	Nil (14)	tr. (19)*	tr. (26)	Nil (26)	Nil (30)	tr. (12)	4:18 (15)	4:20 (20)	3:14 (28)	3:13 (29)	tr. (33)	tr. (12)	4:18 (15)	4:20 (20)	3:14 (28)
80%	Serum	5:18 (15)	5:23 (17)	5:26 (20)	6:28 (21)	6:25 (28)	5:24 (31)	tr.	5:17 (7)	5:26 (9)	5:29 (10)	5:25 (18)	5:23 (21)	tr.	5:17 (7)	5:26 (9)	5:29 (10)
80%	Albumin I (impure)	tr. (15)	5:20 (16)	5:24 (18)	6:25 (21)	5:26 (26)	4:18 (31)	tr. (12)	5:22 (14)	5:26 (14)	5:28 (17)	5:26 (18)	tr. (18)	tr. (12)	5:22 (14)	5:26 (14)	5:28 (17)
80%	10% (w/v) in saline	—	Nil (13)	Nil (20)	Nil (23)	Nil (29)	Nil (33)	—	4:15	4:18 (20)	tr.	Nil (20)	Nil (27)	—	4:15	4:18 (20)	tr.
80%	Albumin II (pure)	—	Nil (12)	Nil (20)	Nil (18)	tr. (23)	tr. (29)	—	—	tr. (20)	tr.	4:22 (24)	tr. (28)	—	—	tr. (20)	tr.
80%	γ-Globulin 10% (w/v) in saline	—	tr. (10)	4:18 (24)	5:21 (26)	4:18 (35)	tr. (42)	4:23 (11)	5:22 (14)	5:24 (19)	3:16 (21)	3:21 (31)	tr. (36)	4:23 (11)	5:22 (14)	5:24 (19)	3:16 (21)
80%	Haemoglobin† 1.25% (w/v) in saline	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Results are the average of 3-7 experiments. A representative selection of these reactions were specifically neutralized by anthrax antiserum.

Conditions of culture see Table 1; inoculum of recently germinated spores: 6×10^7 N.P., 3×10^7 Sterne.

* Occasional batches of tryptic meat broth produced culture filtrates with a significant skin reaction.

† Guinea-pig haemoglobin. A reduction of 1/10 in its concentration did not significantly alter the pattern of toxin production.

maximum toxin content approximately equal to that produced *in vivo*. The toxicity of cultures of the virulent strain is $\frac{1}{2}$ – $\frac{1}{4}$ that of toxic plasma.

The early appearance and rapid disappearance of the toxin in artificial culture probably explains the failure of earlier workers to demonstrate it. In culture, *Bacillus anthracis* organisms change rapidly from toxin producers to toxin destroyers as the result of the production of an intracellular toxin-destroying enzyme system. This change occurs when the bacterial population increases beyond a critical concentration. The appearance of this enzyme was prevented by continuous culture in a mixture of serum + tryptic meat digest where the population and nutritional conditions remained more or less constant; under these conditions, the maximum toxin content of the culture was maintained. Hence, the concentration of toxin does appear to cause the change of organisms from toxin producers to destroyers; the change may be due to a nutritional deficiency. Continuous culture is not unlike the conditions which exist *in vivo*, where any depletion of plasma constituents would be replaced by compensatory mechanisms. One important difference however, is that the bacterial population *in vivo* increases to $c. 1 \times 10^9$ chains/ml. blood, at which concentration complete destruction of the toxin would have occurred *in vitro*. The compensatory mechanisms of the host appears to be able to supply the correct nutritional environment for a toxigenic bacterial population in excess of that supported by normal serum in artificial culture.

We have been unable to identify in detail the nutritional requirements for toxin production and maintenance. The addition of many nutrilites and the use of various stabilizing procedures have not increased the maximal yield of toxin or prevented its destruction. The large molecular components of serum play an important part in toxin production, and it is interesting to speculate whether these components protect the toxin against the action of the toxin-destroying system by acting as competitive enzyme substrates, or whether they provide essential large molecular intermediates for toxin production.

The formation of toxin by the avirulent uncapsulated Sterne strain of *Bacillus anthracis* fits in with our views on the virulence of this organism and on immunity to anthrax. Virulence is due to at least two factors: the toxin and a capsule which contains polyglutamic acid (Smith, Zwartouw & Harris-Smith, 1956). Both factors must be present for full virulence; they both act as aggressins and later in the disease the accumulation of toxin proves fatal to the host. The toxin is the complete natural antigen for stimulating active immunity to anthrax. The Sterne organism lacks a capsule and is therefore almost avirulent, but it is a well-known immunogenic strain, either as a spore vaccine or in the form of a filtrate of cultures grown under special conditions (Belton & Strange, 1954). This strain should therefore produce the toxin in suitable cultures, and this has now been observed. On the other hand, the HM strain is heavily capsulated but since it lacks the capacity to form significant quantities of toxin it is an attenuated strain and non-immunogenic.

Now that the *in vitro* production of the anthrax toxin in serum has been achieved, it should be possible to succeed also with simpler media. The formation of a trace of toxin in tryptic meat digest, which can be increased by the

addition of impure serum albumin or haemoglobin is encouraging in this respect. Furthermore, a product closely connected with the anthrax toxin has already been prepared in a chemically defined medium (Wright, Hedberg & Slein, 1954; Belton & Strange, 1954). It is a non-toxic protective antigen, which on injection leads to the production of antitoxin capable of neutralizing the anthrax toxin from *in vivo* (Smith, *et al.* 1955) or *in vitro* (see above) sources; it also forms common precipitin lines with the toxin in serological diffusion plate analysis (Mr J. L. Stanley and Dr H. Smith, personal communication) and will, to some extent, replace factors I and II in the tests for oedema production and lethality (Smith *et al.* 1956).

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The Role of *Aerobacter* sp., *Escherichia coli* and Certain Amino Acids in the Excystment of *Schizopyrenus russelli*

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SUMMARY: Aqueous extracts of *Aerobacter* sp. and *Escherichia coli* have been found to cause excystment of viable sterile cysts of *Schizopyrenus russelli*. The factors which cause excystment are thermostable. With the aid of paper partition chromatography of the aqueous extract of *Aerobacter* sp., it has been found that part of the excystment-inducing activity is due to the presence of amino acids, some of which have been identified. Amino acids, sugars, purines, pyrimidines, nucleosides, nucleotides and organic phosphates have been tested for their ability to induce excystment. It has been found that some amino acids and a few nucleotides can cause excystment. The effect of pH, concentration and time on excystment with amino acids has been studied.

Bacteria and amoebae live in close association in human and animal intestine, and living bacteria in addition to being the usual food for amoebae are believed to be in some way responsible for the excystment of amoeboid cysts. A study of the amoeba excystment factor or factors produced by common intestinal bacteria might be of significance for more effective chemotherapy of chronic amoebic dysentery. Various factors such as hypo- and hypertonicity of the medium, low oxygen tension, change of pH value, presence of reducing agents or nutrient media, change of temperature, desiccation, presence of bacteria, etc., have been claimed to potentiate the emergence of trophozoites from their resting cysts in Protozoa (see Beers, 1946; Crump, 1950; Hall, 1953; Kudo, 1954; Singh, 1941*b* for the earlier literature). Earlier workers in this field, however, do not appear to have fully recognized the risk of contamination by other organisms inherent in such experiments, especially when nutritionally rich media are to be examined for the presence of excystment factors. Unless aseptic conditions are maintained during experiments, the results will remain open to doubt.

It was shown by Singh (1941*b*) for *Colpoda steinii*, by Beers (1946) for *Didinium nasutum* and by Crump (1950) for Amoeba 4 (*Schizopyrenus russelli*, Singh, 1952) that an environment containing bacteria was necessary for the resting cysts of these Protozoa to excyst. Singh, Mathew & Sreenivasaya (1956) demonstrated that a strain of an *Aerobacter* sp. produced in an actively proliferating culture an excystment factor for *S. russelli* cysts, which was capable of diffusing easily through a collodion membrane; the factor was thermolabile and its activity was somewhat dependent on the pH value of the medium.

The present paper deals with the extraction and isolation of material causing excystment from an *Aerobacter* sp. and from *Escherichia coli* and examines the role of certain amino acids in excystment.

METHODS

Preparation of sterile cysts of Schizopyrenus russelli

The cysts from a 'pure line' culture of amoebae were used in this work (see Singh, 1952, for the characters of *Schizopyrenus russelli* and the culture methods used). The cysts are double-walled and excystment takes place in two stages (Crump, 1950). The amoebae were grown on non-nutrient agar (2.5%, w/v, agar), 0.5% (w/v) NaCl; pH 6.8–7.0; plates supplied with a young culture (3 days old) of *Aerobacter* sp. grown on nutrient agar slopes (Strain 1912; Singh, 1941a). Ten-day-old cysts were collected and washed three times by suspension and centrifugation in sterile distilled water at 500 rev./min. for 3 min., in order to get rid of most of the bacteria. They were then treated with a mixture of penicillin (200 units/ml.) and streptomycin (1000 units/ml.) for 48 hr. at 25° to kill bacteria, centrifuged, and washed with distilled water. The cysts were next treated with 0.5% (w/v) emetine hydrochloride or HCl (1.0–1.5%, w/v) for 24 hr. to destroy active amoebae or partially formed cysts. After washing, these sterile cysts were kept in 0.85% (w/v) NaCl solution at 4° and were used for excystment experiments during 4 weeks, when there was hardly any loss in viability. The freedom of these cysts from bacteria was tested on nutrient agar slopes or in nutrient broth, and their viability by inoculating them in the presence of living *Aerobacter* sp. on non-nutrient agar. The sterile cysts excysted readily in the presence of living bacteria on the non-nutrient agar, although on non-nutrient agar alone there was hardly any excystment.

Method of studying excystment

Petri dishes, each containing a flat-bottom cavity slide placed on a piece of filter paper, were sterilized (160° for 1 hr.). A very tiny drop (c. 0.001 ml.) of cyst suspension, containing 50–125 cysts, was transferred to the centre of the cavity slide by a finely drawn capillary pipette. A small drop of the test fluid (c. 0.005–0.01 ml.) was then added and the filter paper was moistened with sterile distilled water to prevent evaporation of the liquid in the cavity slide. These Petri dishes were then incubated at 24–25° for 18–24 hr. After this period, the cavity of the slide was sealed with a sterile coverslip, the slide inverted carefully and examined under the low power of a microscope. The fluid containing the cysts remained as a drop and did not spread out. A cyst was considered excysted only when an amoeba escaped from it and was found to be moving freely in the surrounding medium. The percentage excystment was calculated from the count of the amoebae and the unexcysted cysts. As there was no food supply for the excysted amoebae, they remained viable for 72–96 hr. and then died or tried to encyst. To ascertain whether sterile conditions had prevailed during the experiment, the fluid from the cavity slides was drawn out at the end of the experiment and was inoculated into nutrient broth or on nutrient agar.

Preparation of the aqueous extracts of bacteria

(1) *Aerobacter* sp., *Escherichia coli* and *E. coli* strain RCI (phage-resistant strain of *E. coli*; Gupta, 1957) were grown in a liquid defined medium (NH_4Cl , 1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g.; KH_2PO_4 , 1.5 g.; Na_2HPO_4 , 3.5 g.; lactic acid, 9.0 g.; distilled water, 1000 ml.; pH 6.8–7.0) at 37° for 17 hr. The organisms were washed thrice with distilled water by centrifugation. They were then killed by suspending them in ice-cold acetone for 30 min., washed once with acetone and dried in a vacuum desiccator. The dried organisms were ground with sterile glass dust and distilled water in a mortar, and the water-soluble portion collected by centrifugation. This aqueous extract was sterilized at 15 lb./sq.in. pressure for 15 min. and had a pH value of about 6.8–7.0. The bacterial residue was washed three times and suspended in distilled water.

(2) The washed *Aerobacter* sp. (17 hr. old) was suspended in distilled water, heated at 100° for 20 min. in a water bath (Freeland & Gale, 1947) and centrifuged. The supernatant liquid was at pH 6.5. The organisms were washed three times and suspended in distilled water.

RESULTS

Effect of aqueous extracts of bacteria on excystment

The excystment properties of the aqueous extract and of the bacterial residue suspension of the *Aerobacter* sp., *Escherichia coli*, *E. coli* strain RCI, distilled water and normal saline were tested in duplicate; the results are given in

Table 1. *The effect of various preparations on excystment*

Preparation	Duration of storage at 4° (days)				
	1	3	5	7	14
	Degree of excystment (%)*				
Extract of:					
<i>Aerobacter</i> sp.	96	92	95	85	87
<i>Escherichia coli</i>	93	90	—	83	76
<i>E. coli</i> strain RCI	96	—	93	—	—
Bacterial residue suspended in distilled water	Nil	Nil	Nil	Nil	Nil
Distilled water (control)	Nil	1.5	Nil	Nil	Nil
Normal saline	Nil	Nil	2.6	Nil	Nil

* Means of duplicate set.

Table 1. The variation in the % excystment of duplicate sets was very little. The fact that the aqueous extract showed marked ability to induce excystment while the bacterial residue suspension was unable to do so, indicated that the bacterial products which induce excystment are water soluble. Storage of the extracts up to a period of 14 days at 4° or autoclaving at 15 lb./sq.in. for 20 min. had practically no adverse effect on excystment.

It is known (Singh, 1946) that cysts produced on different occasions under

similar cultural conditions may vary in their % viability. Therefore, it is important to know the % viability of a batch of cysts before using them in excystment experiments. The ability of the extracts of *Aerobacter* sp. and of *Escherichia coli* to cause a high % excystment provided a useful method of finding out the % viability in a particular batch of cysts.

Effect of pH value of a bacterial extract on excystment

The extract of acetone-killed *Aerobacter* sp. (pH. 6.8) was adjusted to different pH values between 2 and 8 by adding HCl or NaOH. There was no excystment at pH 2-3; pH 6-8 was found to be most favourable range for the excystment of *Schizopyrenus russelli*.

Characterization of excystment-causing factors from the water-soluble extract of Aerobacter sp.

The water-soluble extract (W) of *Aerobacter* sp. which showed 93 % excystment, obtained from organisms killed by acetone, was evaporated to dryness below 50°, under reduced pressure. It was extracted with 80 % (v/v) ethanol in water and centrifuged. The insoluble residue (R) when dried and dissolved in distilled water, showed a greatly decreased ability to cause excystment. The ethanolic solution (E) was evaporated to dryness and the residue dissolved in distilled water; it was very effective in causing excystment. This solution showed a strong positive ninhydrin reaction, a positive test for sugars (aniline hydrogen phthalate) and a positive test for organic phosphate. This material was fractionated by paper partition chromatography. The solution was applied to a sheet of Whatman No. 1 filter paper as a streak and the paper developed with butanol + acetic acid + water (4:1:5) as the solvent system by the descending technique. The solvent was allowed to travel to the edge of the paper, the paper dried and a narrow segment of the chromatogram cut from one side and sprayed with ninhydrin for the location of the amino acids. Nine distinct ninhydrin-positive spots were visible. The chromatogram was then divided into twenty bands, ten corresponding to the ninhydrin-positive bands and ten other bands. These bands were excised, eluted with water and the eluates autoclaved and tested for their excystment properties. Six of these fractions showed excystment activity; five of these corresponded with the five slower-moving ninhydrin-positive bands. The sixth fraction (R_f 0.53-0.63) with a ninhydrin-negative zone; this fraction has not yet been identified.

Paper chromatography of the aqueous extract (W) was repeated with the same solvent system. To obtain better resolution of the amino acids, the chromatogram was allowed to run 30 hr. and a segment of the paper developed with ninhydrin as before. Again nine strong and two weak ninhydrin-positive spots were obtained. The bands corresponding to the nine distinct ninhydrin-positive spots were marked on the chromatogram, the bands excised, eluted with water and the eluates tested for the excystment activity. Table 2 gives a summary of the results, the R_f value of the fractions and the corresponding % excystment obtained. It is clear from Table 2 that only the six slow-moving fractions showed excystment activity; of these, fractions one and five

were most active. The inability of the other fractions to cause excystment may have been due to a low concentration of these amino acids. The amino acids identified in these fractions by two-dimensional chromatography were cystine, lysine, arginine, histidine, aspartic acid or glycine, glutamic acid, alanine, tyrosine, valine, isoleucine or phenylalanine (Table 2). The solvent systems used were butanol + acetic acid + water (4:1:5) and phenol + water + ammonia (80:20 with 3% ammonia). Further work to characterize the excystment-causing material insoluble in 80% (v/v) ethanol in water and the determination of the relative concentration of amino acids is in progress.

Table 2. *Excystment caused by amino acids isolated from the water-soluble extract of Aerobacter sp. by paper chromatography*

Fractions	R _i * in BuOH:AcOH:H ₂ O			pH value	Identified amino acid	Degree of excystment† (%)
	4	1	5			
1		0.140		6.0	Cystine	67
2		0.187		6.5	Lysine	39
3		0.238		6.5	Arginine/histidine	40
4		0.355		6.0	Aspartic acid or glycine	19
5		0.410		6.0	Glutamic acid	66
6		0.477		6.0	Alanine	7
7		0.618		6.0	Tyrosine	Nil
8		0.79		6.5	Valine	Nil
9		1.00		6.0	Isoleucine or phenylalanine	Nil

* R_i refers to the distance travelled by individual fractions from the origin as compared to that of isoleucine taken as 1.

† Mean of duplicate sets.

The finding that the bacterial products of the *Aerobacter* sp. which caused excystment were water soluble and that part of this activity was due to amino acids, led to the testing of various pure amino acids for their excystment inducing activity. Certain sugars, purines, pyrimidines, nucleosides, nucleotides, and vitamins B₁, B₂, B₆ were also examined.

Effects of various amino acids on excystment. The excystment inducing activity of twenty-three amino acids dissolved in distilled water and autoclaved was tested (Table 3). Some of them induced good excystment, while others induced poor or no excystment. Since the low pH value of the aqueous extract of *Aerobacter* sp. was a limiting factor and pH values between 6 and 8 were most favourable for excystment, some of the amino acids were tested at different pH values. Solutions of DL-histidine dihydrochloride, DL-lysine dihydrochloride, L-cysteic acid, L-histidine and glutathione, which gave no excystment at pH 1.0–3.0 gave good excystment at pH 7. There were, however, some amino acids which caused poor or no excystment even in the favourable pH range of 5.0–7.0 (Table 3).

To study the effect of the concentration of amino acids on excystment, some of the amino acids were tested at different concentrations (pH 6–7); the results are recorded in Table 4. It was found that a definite concentration

was necessary to give good excystment. There was a certain degree of variation in % excystment when cysts from different batches were tested with the same amino acids. Therefore, to get comparative % excystment, different substances should be tested with the same batch of cysts.

Table 3. *The effect of various amino acids on excystment*

Amino acid	Concentration	pH	Degree of excystment* (%)
DL-Valine	2.0 % (w/v)	6.0	68
DL-Serine		6.8	69
DL-Methionine		6.0	52
Glycine		6.0	45
DL-Histidine dihydrochloride		1.0	Nil
DL-Threonine		5.0	49
DL-Proline		7.0	47
DL-Isoleucine		6.5	53
DL-Lysine dihydrochloride		1.0	Nil
DL- β -Phenylalanine		6.0	27
L-Asparagine		5.0	Nil
L-Glutamic acid		6.0	61
DL-Aspartic acid		3.0	Nil
DL-Leucine		6.5	7
DL- α -Alanine		6.0	15
L-Hydroxyproline	5.0 % (w/v)	7.2	76
L-Cysteic acid		1.0	Nil
L-Histidine		1.0	Nil
L-Arginine		7.0	25
DL-Ornithine HCl		6.0	2
Glutathione		3.0	Nil
DL-Alanyl-D-asparagine		6.0	9
Sarcosine		5.0	Nil

* Mean of duplicate sets.

Table 4. *The effect of the concentration of amino acids on excystment*

	Dilutions			
	2.0 % (w/v)	1.0 % (w/v)	0.2 % (w/v)	0.1 % (w/v)
	Degree of excystment* (%)			
L-Glutamic acid	95	88	—	2
DL-Histidine	88	83	13	Nil
DL-Isoleucine	—	48	57	20
L-Cysteic acid	67	75	25	9
DL-Lysine	—	65	10	3

* Mean of duplicate sets

Effect of sugars on excystment. 1.0 and 0.5 % (w/v) aqueous solutions of arabinose, maltose, raffinose, glucose, lactose, ribose and glucosamine were sterilized by autoclaving (15 lb./sq.in. for 20 min.) and tested for their excystment properties. There was no excystment in the presence of these compounds.

Effect of purines, pyrimidines, nucleosides, nucleotides and some organic phosphates on excystment. As certain purines, pyrimidines, nucleosides and nucleotides or organic phosphates might be present in the free state in the metabolic pool in the bacteria, 1.0% (w/v) solutions of uracil, uridine, cytidine, adenosine, uridylic acid, guanylic acid, yeast and muscle adenylic acids, and 0.5% (w/v) solutions of adenosine triphosphate, β -glycerophosphate, fructose-6-phosphate, glucose-1-phosphate, potassium metaphosphate and 0.2% (w/v) solutions of cytosine, hypoxanthine and adenine were tested for their ability to induce excystment. Only muscle adenylic acid, adenosine triphosphate and fructose-6-phosphate induced excystment of 35, 65 and 42% respectively; the other compounds were ineffective. Muscle adenylic acid was tested at 2.0, 1.0, 0.2 and 0.1% (w/v) concentrations and the percentage excystment of these was 42, 35, 9 and 3%, respectively, showing that higher concentrations caused better excystment.

Effect of vitamins on excystment. Samples of vitamins B₁, B₂ and B₆ were dissolved in distilled water, autoclaved and tested for excystment properties. Only vitamin B₁ at a dilution of 1.0% (w/v) induced excystment.

Excystment of Schizopyrenus russelli cysts at different intervals in the presence of excystment inducing agents. 1.0% (w/v) solution of L-glutamic acid, L-cysteic acid and aqueous extract of acetone-killed *Aerobacter* sp. at pH 7.0 were used in this experiment, keeping distilled water as control. In the presence of the bacterial extract the excystment was somewhat more rapid in the first few hours, and at the end of 24 hr. all the viable cysts had excysted. The excysted amoebae remained viable in amino acids during 96 hr. without multiplying or encysting.

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Bacteriological Changes in Silage Made at Controlled Temperatures

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SUMMARY: Silage made in the laboratory at 22°, 30° and 40° from five specimens of grass was examined after 1, 2, 3 and 8 days and 6 months. The dominant bacteria on fresh grass were obligate aerobes which died rapidly in a closed silo. Bacteria capable of anaerobic growth were represented irregularly and often weakly on grass. This can account for much of the variation in the composition of bacterial populations in silage. Organisms that developed extensively in much of the silage were the *Klebsiella* group, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Clostridium* and *Bacillus*. Each proceeded to multiply soon after silos were closed; all stopped growing at about the same time. The rate of growth and the concentration of viable organisms reached were determined by properties of the herbage. Gram-negative organisms were restrained at 40°, *Clostridium* and *Bacillus* at 22°. The multiplication phase was short; it could be completed by the third day at all three temperatures. Cessation of growth could not be attributed to the accumulation of acids. Much acid was formed after all the main groups had reached the phase of decreasing viable count. When the lactic acid fermentation was not vigorous the decreases in pH value were most rapid at 40° and slowest at 22°.

In the commonest methods of making silage the preservation of the herbage is known to rely in part on the formation of acids, chiefly lactic acid, by bacterial fermentation of plant sugars (Watson, 1939; Barnett, 1954). When sufficient acid is produced the material will be preserved, provided that oxygen is excluded. Inadequate concentrations of acid may allow an intense decomposition in which clostridia appear to be the chief causative agents; access of air permits the activity of aciduric aerobes. All the genera of lactic acid bacteria now recognized, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Pediococcus*, have been demonstrated in silage. In many instances lactobacilli were found to be the dominant bacteria. Coliform organisms, *Clostridium* and *Bacillus* spp. have also been shown to multiply in much silage. Other bacterial groups and yeasts have been detected less often. Most investigators agree that in the early stages there is a multiplication of a mixture of organisms, including the coliform group, and that in well-preserved silage lactic acid bacteria, especially lactobacilli, become dominant. Apart from these features the literature indicates that there is much variation in the course of bacterial development.

In agricultural practice much importance is attributed to temperature as a factor influencing the silage fermentation, yet work on the relationship of temperature to the efficiency of preservation has yielded surprisingly contradictory results. It is therefore remarkable that little attention has been given

to the effect of temperature on the bacterial populations, except indirectly by chemical analyses of their metabolic products. Among the few contributions on this problem are those of Burri (1918), Gerlach, Günther & Seidel (1926) and Scheunert & Schieblisch (1926), who made observations on the types of bacteria which develop during the heating of silage. Their findings lack uniformity and allow of little generalization. The results of an experiment comparing the changes in viable bacterial count of silage made at 22°, 30°, and 40° were given by Stirling (1951).

The object of the work about to be described was to gain a clearer understanding of the bacterial ecology of silage. An attempt was made to follow the development of the several bacterial groups when the conditions were varied (1) by using specimens of rye-grass which differed in character and (2) by making the silage on each occasion at 22°, 30° and 40°. In order to control temperature the silage was prepared in the laboratory.

METHODS

Grass. The herbage used for ensilage was perennial rye-grass (*Lolium perenne*), either S23 or S24. The grass was grown as pure strains on small plots. Particulars of the specimens employed are shown in Table 1. In all manipulations of the grass it was not allowed to come in contact with unsterile surfaces. The harvesters wore sterile rubber gloves and cut the grass with sterile sheep shears. The herbage was collected in bags, and without delay taken to the laboratory where it was cut into short lengths (1–3 cm.) and thoroughly mixed.

Table 1. *The perennial rye-grass used for ensilage*

Experi- ment	Type of grass	Date of cutting	Dry matter (%)	Crude protein* (% of dry matter)
7	S24, spring growth, mainly leaf, first cut	22 May	21.1	14
8	S24, preflowering, first cut	12 June	21.4	8.1
9	S24, stemmy, second cut	20 Aug.	25.6	9.0
10	S23, summer-sown, first cut	28 Aug.	21.0	10.8
15	S24, spring growth, mainly leaf, first cut	9 June	20.2	21.4

* N × 6.25.

Ensilage. Pyrex glass tubes c. 20 × 3 cm. were filled each with 50 g. grass. Using a wooden rod of a diameter slightly less than that of the tube the grass was packed as evenly as possible. Each tube was closed with a rubber stopper carrying a valve of mercury supported on a sintered glass disk, which permitted the escape of gases but prevented the entry of air. Before use the stoppers were sterilized over formalin in a desiccator. A set of filled tubes was

placed, in the dark, in each of three water baths controlled at 22°, 30° and 40°.

Preparation of grass and silage for examination. A sample (50 g.) of the fresh grass, taken after chopping and mixing, was placed in a tared macerator jar. Sterile water was added until the suspension weighed 300 g. The material was then disintegrated for 2 min. by an electric top-drive macerator. In the case of silage all the material was withdrawn from a tube by a sterile corkscrew with an elongated shank and treated in the same way as the grass. The macerate was used for the bacteriological examinations and for the electrometric determination of pH value.

The macerator was washed thoroughly between each examination and sterilized by swabbing with ethanol and flaming. The rubber pad surrounding the spindle was difficult to sterilize; it was covered before each maceration with a sterile disk of rubber sheeting slotted at the centre so that it fitted closely round the spindle. Sterility tests, which were carried out regularly by running the macerator with 300 g. sterile water and then plating 1 ml. of the water on each of several media, rarely yielded more than an occasional colony when these precautions were taken.

Bacteriological media and methods

Serial decimal dilutions of the macerate were made in sterile tap water and were used for the inoculation of five media which yielded counts of different bacterial groups. The media and their uses are described below.

Medium (1). Glucose yeast agar: peptone (Evans), 1 g.; meat extract (Lemco), 1 g.; yeast autolysate, 5 ml.; glucose, 0.5 g.; agar (Davis), 1.5 g.; tap water, 100 ml.; pH 6; sterilized momentarily up to 22 lb./sq.in. To prepare the yeast autolysate, 1 kg. brewer's yeast in 1 l. water was held at 50° for 24 hr.; after centrifugation, the supernatant fluid was sterilized in bottles momentarily at 22 lb./sq.in.

Poured plates were incubated in air for 5 days at 30°. The medium might be expected to support the growth of most of the silage organisms other than the obligate anaerobes.

Medium (2). Acetate agar (Keddie, 1951): peptone (Evans), 1 g.; meat extract (Lemco), 1 g.; yeast autolysate (as in medium 1), 5 ml.; tomato extract, 20 ml.; glucose, 1 g.; Tween 80, 0.05 ml.; agar (Davis), 1.5 g.; tap water, 100 ml. The peptone, meat extract and agar were dissolved together, the yeast autolysate and tomato extract then added and the pH value adjusted to 5.4; after filtration the glucose and Tween 80 were added, and the medium bottled and sterilized at 15 lb./sq.in. for 15 min. To prepare the tomato extract, 1 kg. tomatoes + 450 ml. water were steamed for 30 min.; the pulp was then squeezed through muslin.

Just before pouring plates, 10 ml. of 2M-acetic acid/sodium acetate buffer of pH 5.4 were added to 90 ml. of molten medium. The final pH should be 5.4 ± 0.05 . After the inoculated agar had solidified a second layer of the acetate agar was added. Plates were incubated in air for 5 days at 30°. They were used for the enumeration of lactobacilli. For this purpose it was essential to

identify colonies of *Lactobacillus* by isolation and microscopical examination of the isolates, since *Leuconostoc* and *Pedicoccus* are capable of growth on the medium.

Medium (3). Lactate agar: ammonium lactate syrup (c. 61%), 1 ml.; glucose, 0.2 g.; L-glutamic acid, 0.2 g.; K_2HPO_4 , 0.1 g.; $MgSO_4 \cdot 7H_2O$, 0.02 g.; washed shred agar, 1.5 g.; tap water, 100 ml.; pH 6; sterilized momentarily at 22 lb./sq.in. Just before use crystal violet was added to a final concentration of 1/500,000 in order to inhibit Gram-positive organisms. On plates incubated for 3 days at 30° a count is made of Gram-negative bacteria.

Medium (4). Medium for lactate-fermenting anaerobes (Rosenberger, 1951): sodium lactate syrup (c. 70%), 1.5 ml.; sodium acetate, hydrated, 0.8 g.; $(NH_4)_2SO_4$, 0.05 g.; yeast autolysate (as in medium 1), 3 ml.; biotin, 0.01 μ g.; *p*-aminobenzoic acid, 10 μ g.; L-cysteine hydrochloride, 0.05 g.; thioglycollic (mercaptoacetic) acid, 0.05 ml.; a mineral supplement; resazurin, 0.5 mg.; agar, 0.2 g.; tap water, 100 ml.; pH 6; sterilized momentarily at 22 lb./sq.in. The agar was dissolved before adding the other ingredients.

Medium (5). Medium for proteolytic anaerobes (Rosenberger, 1951): peptone (Evans), 1.5 g.; gelatin, 12 g.; yeast autolysate (as in medium 1), 1 ml.; L-cysteine hydrochloride, 0.05 g.; resazurin, 0.5 mg.; tap water, 100 ml.; pH 7; sterilized momentarily at 22 lb./sq.in.

Before inoculation of media (4) and (5) the tubes were placed in boiling water for 10 min. and then cooled to 45°. The inoculum was planted into the depths of the medium; the introduction of air and mixing was avoided. After inoculation the tubes were chilled to solidify the agar or gelatin. The medium was then covered to a depth of 3 cm. with a seal of: agar, 1 g.; L-cysteine hydrochloride, 0.05 g.; thioglycollic acid, 0.05 ml.; resazurin, 0.5 mg.; tap water, 100 ml.; pH 7; sterilized momentarily at 22 lb./sq.in.

The cultures were incubated in air for 7 days at 30° or 37°. In the first experiments 37° was used; as a precaution 30° was adopted. Up to now there have been no indications of different results at the two temperatures. The incubated cultures were examined as follows.

(a) Lactate fermentation in medium (4) was recognized by gas formation, sufficient to raise the seal at least 2 cm., and by an increase in pH value. To detect changes in pH value some of the culture was withdrawn and tested with bromothymol blue on a spot-plate.

(b) Protein breakdown in medium (5) was shown by gelatin liquefaction and by strong reactions for NH_3 and H_2S in spot tests with Nessler's reagent and lead acetate. A vanillin spot-test (Roessler & McClung, 1943) assisted the recognition of *Clostridium sporogenes*. Some, but apparently not all, of these reactions may be produced by non-proteolytic clostridia and by certain other bacteria (Rosenberger, 1956).

Dilutions of the silage macerate were inoculated into media (4) and (5) in triplicate, and the most probable numbers of each type of anaerobe were estimated by using the table of Hoskins (1934).

RESULTS

The bacterial populations of the fresh grass

The data in Table 2 show that the bacterial populations on the specimens of grass varied considerably. In all cases the dominant organisms were strict aerobes. Many were Gram-positive and coryneform. The Gram-negative aerobes were in part pseudomonads, some of which were fluorescent, and in

Table 2. *Bacterial populations of the fresh grass*

Experiment	Counting medium used			
	Glucose yeast agar	Lactate agar	Acetate agar	Media for anaerobes
	Count/g. dry-wt. grass			
7	9,000,000	2,100,000	< 25	< 170
8	61,000,000	1,500,000	< 25	< 17
9	220,000,000	170,000,000	22	2000
10	35,000,000	110,000,000	5000	50
15	2,600,000	< 100,000	29	2000

part non-motile forms. The majority of the aerobic organisms formed colonies which were some shade of yellow. Bacteria capable of anaerobic growth, which have greater significance in relation to silage, were much less numerous on the fresh grass. Among the Gram-negative organisms estimated on lactate agar, facultative anaerobes of two kinds were detected as minorities. They were (a) a diverse group possessing the general characters of *Klebsiella*, and (b) the organism which has most frequently been named *Bacterium herbicola* (reviewed by Mack, 1936). Lactobacilli, as shown by the counts on acetate agar, were extremely scarce except in Expt. 10; in two cases this group was not demonstrated in 1 ml. of undiluted grass macerate. The counts of clostridia were little higher; the species isolated were *Clostridium butyricum*, *C. welchii* (*C. perfringens*) and *C. bifermentans*. Aerobic spore formers were likewise scarce and did not occur in the macerate at dilutions greater than 1/10. *Bacillus licheniformis* was isolated from every specimen of grass; *B. subtilis*, *B. pumilus* and *B. circulans* were encountered less often. The methods employed, although they did not afford counts of lactic acid cocci, were sufficient to prove that these organisms were rare. In some cases streptococci were detected by means of selective methods in 1 ml. of undiluted macerate.

The literature on the bacteria which occur on the aerial parts of growing plants is extensive but much of it is restricted in scope. It is of immediate interest that in work with several crops, results similar to those just described have been reported: for aerobes and coliform organisms by Kroulik, Burkey & Wiseman (1955); for clostridia by Scheunert & Schiebllich (1926), Ruschmann & Harder (1931), Allen & Harrison (1937) and Nilsson & Nilsson (1956); for lactic acid bacteria by Ruschmann & Koch (1930), Stirling (1953), Kroulik, Burkey & Wiseman (1955) and Nilsson & Nilsson (1956). The counts of lactobacilli

reported by Allen, Harrison, Watson & Ferguson (1937) and of lactic acid bacteria by Arnaudi (1940) are so high in comparison with all the other findings as to indicate that they were not obtained on freshly harvested herbage.

Changes in the total viable count after ensilage

The aerobic and anaerobic counts made in the silage have been combined to give total viable counts which are shown in Fig. 1. In Expt. 8 the results were incomplete for the silage held at 30° for 2 days and at all three temperatures

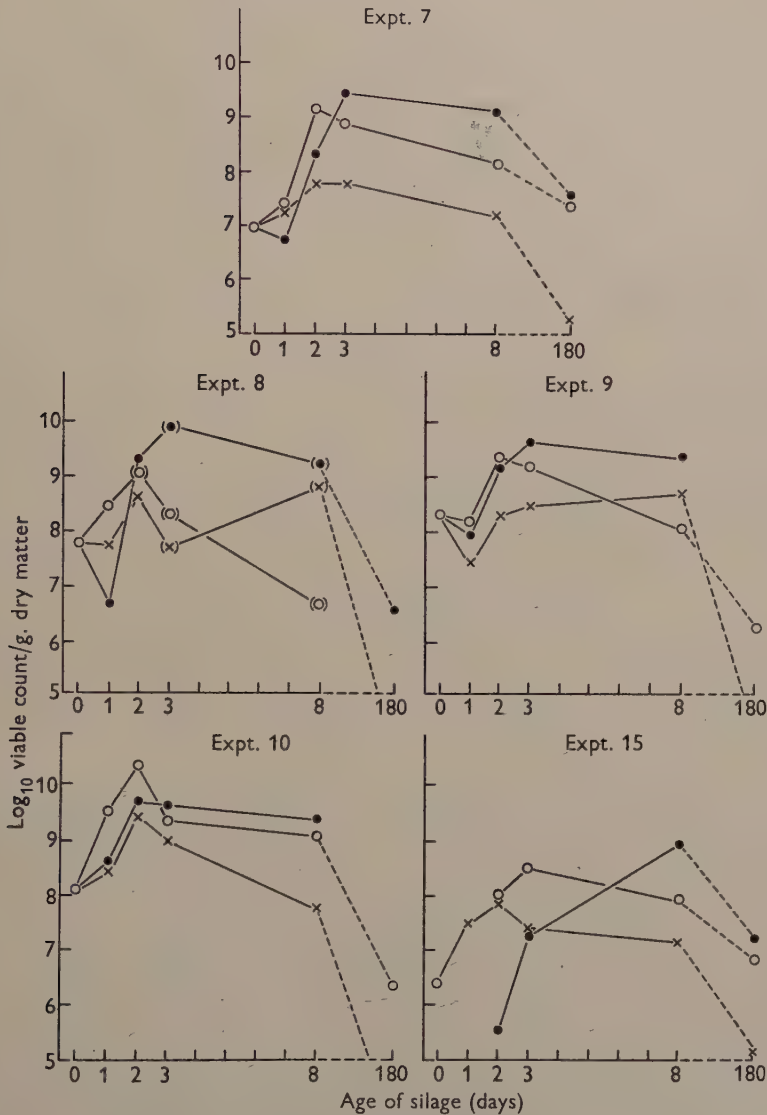


Fig. 1. Counts of total viable bacteria in silage. Silage made at 22°, ●—●; 30°, ○—○; 40°, ×—×. Incomplete count, —()—.

for 3 and 8 days. In these instances faulty batches of media (4) and (5) were used, and the counts shown in Fig. 1 either do not include any anaerobes or do not include lactate-fermenting anaerobes. The figures for the 180-day examination of the 30° silage in Expt. 8 and for the 22° silage in Expts. 9 and 10 have been omitted as in each case air had entered the tube, mould had developed, and the viable counts were much higher than in anaerobic silage. In Expt. 15 growth was unexpectedly slow, and the dilutions examined on the first day were not all sufficiently small to afford complete results.

When living herbage is packed in a container which excludes air the oxygen is quickly consumed and replaced by CO₂. The efficiency of the method of cultivating obligate anaerobes which utilizes this action (McClung, McCoy & Fred, 1935) shows how rapidly will the process be completed when the gas space is small. In the laboratory silage this change was accompanied by a rapid disappearance of the aerobic bacteria of the herbage. At the same time several types of bacteria which are equipped for anaerobic growth proceeded to multiply rapidly. Those that did so had been minorities, frequently very small minorities, in the bacterial populations of the fresh herbage. The net result was that the viable count could for a period show a decrease from the original value. Several examples of this may be seen in Fig. 1. The graphs show that the rate of growth and the maximum density of the viable populations varied with the herbage and with the temperature. It might be expected that the results were influenced in some measure by the variation, to be discussed presently, in the types of bacteria which developed. The multiplication phase was frequently of short duration. In Expt. 10 it was completed by the third day even at the lowest of the three temperatures. It is evident that a count of bacteria after 6 months—i.e. when a field silo might be opened—gives little information concerning the course of the bacterial development.

The bacteria that multiplied in the silage

An attempt was made to fractionate the complex populations which developed in all the silage. A primary division was made into four fractions, and the results are shown in Table 3. Additional details are given in the following discussion.

Gram-negative organisms. The lactate agar (medium 3), used for the estimation of these organisms, was sufficiently selective for the purpose. Gram-positive bacteria could develop on the low-dilution plates required in the examination of aged silage and also in the vicinity of colonies of Gram-negative rods, but they were usually recognized without difficulty.

With the exception of a few examinations the dominant Gram-negative organisms in the silage were indentified with the *Klebsiella* group. On the lactate agar they formed mucoid unpigmented colonies. They showed variations in their action on lactose (acid and gas, acid only, delayed fermentation and no change, in tests at 30°), glucose (acid and gas or acid only), gelatin and casein, and in their ability to grow at 37°. These variations did not appear to be correlated. Organisms which show a delayed fermentation of lactose are possibly responsible in part for the erratic and indefinite nature of the results obtained

Table 3. *Bacterial groups in silage made at 22°, 30° and 40°*

	Silage at 22° Age (days)					Silage at 30° Age (days)					Silage at 40° Age (days)				
	1	2	3	8	180	1	2	3	8	180	1	2	3	8	180
	Count (millions/g. dry wt. original grass)					Count (millions/g. dry wt. original grass)					Count (millions/g. dry wt. original grass)				
Expt. 7															
Gram-negative	3.5	170	2300	660	<0.1	17	1600	380	18	<0.1	0.3	<0.1	<0.1	<0.1	<0.1
Anaerobes	<0.1	0.1	0.5	3.8	32	<0.1	4.0	16	20	24	14	1.1	1.1	0.1	0.1
Lactobacilli	n.c.	n.c.	n.c.	n.c.	n.c.	<0.1	3.0	n.c.	n.c.	n.c.	n.c.	0.3	2.1	9.0	<0.1
Remainder	1.9	13	560	520	1.1 B	4.7	9.0 B	440	130	2.5 B	4.6	43 B	61	6.6 B	<0.1
Expt. 8															
Gram-negative	2.6	1200	6600	83	<0.1	280	350	150	<0.1	—	2.5	<0.1	<0.1	<0.1	<0.1
Anaerobes	<0.1	<0.1	—	—	2.4	2.4	—	—	—	—	37	<0.1	—	—	<0.1
Lactobacilli	<0.1	<0.1	<0.1	270	n.c.	<0.1	<0.1	<0.1	<0.1	—	<0.1	<0.1	<0.1	<0.1	<0.1
Remainder	1.4	450	2000	1700	1.5	<0.1	700	33	5.5	—	12	540 B	5.3	850 B	<0.1
Expt. 9															
Gram-negative	100	1400	2000	2400	—	150	1500	1400	140	<0.1	0.8	0.4	0.5	11	<0.1
Anaerobes	<0.1	<0.1	<0.1	<0.1	—	<0.1	1.3	0.2	0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1
Lactobacilli	n.c.	n.c.	1.4	n.c.	—	1.1	29	n.c.	69	2.1	25	220	220	76	<0.1
Remainder	0.1	160	1700	3.6	—	1.0	630	22	<0.1	0.3	2.0	<0.1	140	590	<0.1
Expt. 10															
Gram-negative	210	3400	170	150	—	2400	2600	760	1.4	1.7	320	400	270	<0.1	<0.1
Anaerobes	<0.1	<0.1	<0.1	<0.1	—	2.5	0.5	0.1	<0.1	<0.1	0.3	0.3	0.3	<0.1	<0.1
Lactobacilli	44	1400	1000	1100	—	2800	2100	1500	1200	<0.1	43	320	490	28	<0.1
Remainder	200	<0.1	2800	1300	—	<0.1	18000	500	100	0.4	68	3100	260	38	<0.1
Expt. 15.															
Gram-negative	<0.3	<0.3	11	870	<0.1	<0.3	19	78	1.0	<0.1	<0.3	<0.1	<0.1	<0.1	<0.1
Anaerobes	<0.1	<0.1	<0.1	<0.1	2.5	<0.1	14	5.6	16	6.7	5.1	71	6.7	9.5	0.1
Lactobacilli	<0.1	n.c.	0.1	<0.1	0.3	0.1	4.4	13	8.5	1.5	<0.1	0.9	3.0	0.4	<0.1
Remainder	—	—	12	84	14 B	0.3	64 B	170 B	73 B	1.5	29 B	8.1 B	14 B	4.6 B	<0.1

n.c., no confirmation that lactobacilli were represented on acetate agar plates; —, data incomplete; B, *Bacillus* spp. dominant.

when the dilution method of counting, using liquid lactose media, is applied to silage. *Escherichia coli* was detected on three occasions only, all in Expt. 10, and at no time did it comprise more than 10% of the Gram-negative population. *Bacterium herbicola* occurred commonly, but it rarely represented more than 10% of all the Gram-negative rods in the silage. On lactate agar its colonies are distinguishable by their yellow colour. One other organism having yellow colonies was detected infrequently. It differs from *B. herbicola* in forming acid from lactose, in producing indole and in other properties.

During the phase of active growth the Gram-negative organisms dominated the bacterial populations, in terms of plate count, when the silage temperature was 22° and in three cases when it was 30°. In the silage at 40° they competed less effectively with other groups, a result which may be related to the finding that the maximum temperature for some strains is below 37°. Generally, the Gram-negative rods were the first of the main bacterial groups to show loss of viability after active multiplication stopped. One anomalous result was their presence in Expt. 10 after 6 months at 30°, when lactic acid bacteria had disappeared and the pH value had reached 4.2. The isolates in this case were recognized as belonging to a species which was not normally detected but they were not properly characterized. They were shown to be strict aerobes. Hence it is thought that they probably represented survivors of the original grass population rather than organisms which had multiplied in the silage.

Anaerobes. In most examinations the dominant species were either lactate fermenters or actively proteolytic. With the exception of the lactate-fermenting, Gram-negative coccus *Veillonella gazogenes*, which was isolated from silage made at 40°, all the anaerobes detected in these experiments were clostridia. Of the lactate-fermenting clostridia, *Clostridium butyricum* and an organism similar to *C. paraputrificum* (atypical in lacking motility and in some fermentation tests) were capable of active multiplication in the first 24 hr. at 40°. The latter organism was no longer detectable on the eighth day in the silage at any temperature. The actively proteolytic species were *C. bifermentans*, which multiplied in the first 24 hr. at 40°, and *C. sporogenes*, which tended to appear more slowly. Of these, only *C. sporogenes* was found at 6 months. Other species of clostridia were shown to have multiplied in single experiments. They are *C. tetanomorphum*, which ferments lactate but fails to grow satisfactorily in the selective lactate-acetate medium, and two species which are inactive towards lactate, *C. welchii* (*C. perfringens*) and *C. sphenoides*. These organisms were demonstrated by means of the gelatin medium in cases where they were not outnumbered by the actively proteolytic species.

The temperature of the silage was not found to determine the species of clostridia which developed, and neither the lactate-fermenting nor the actively proteolytic group consistently outnumbered the other at any of the three temperatures used. For these reasons the counts derived from media (4) and (5) have been combined to give total counts of anaerobes; these are set out in Table 3. The regular application of confirmatory tests excludes the possibility of a single organism having been responsible for a simultaneous count in both

media. The results show that the main effects of temperature on the anaerobes were (1) at 22° the lag phase was long and the growth slow, (2) at 40° growth usually started immediately and the phase of decline set in early.

This work has been sufficient to indicate that the conditions which control spore germination, growth and spore formation of clostridia in silage are distinctly intricate. In addition, it has been shown that an examination of aged silage may give no information about the growth of these organisms at an earlier period. This last conclusion is in essential agreement with the findings of work in which the butyric acid content of mature silage was compared with its clostridial count (Ruschmann & Harder, 1931; Kreula, 1955; Nilsson, Nilsson & Abrahamson, 1956).

Lactobacilli. The data for this group were derived from the acetate agar plates. Other silage organisms that develop on the medium are *Leuconostoc* and *Pediococcus*; on rare occasions yeasts have appeared. To permit the recognition of lactobacilli isolations were made from a sample of the colonies on a suitable plate. The counts of lactobacilli shown in Table 3 are based on the proportionate representation of these organisms among the isolates. Where none of the colonies was that of a lactobacillus no figure is given in the table. The data are subject to large errors yet they do provide information on a bacterial group which is believed to have a special influence on the silage fermentation.

The pure cultures of lactobacilli were examined in varying degrees of detail. Of the homofermentative isolates that were extensively characterized, most were identified as *Lactobacillus plantarum* and *L. acidophilus*. Less frequently isolated were *L. casei* and other groups which do not appear to have been clearly defined by previous work. *L. plantarum* was encountered much less regularly than would be expected on the basis of the information in the literature (Allen & Harrison, 1936; Allen *et al.* 1937; van Beynum & Pette, 1939; Arnaudi, 1940; Orla-Jensen, Orla-Jensen & Kjaer, 1947; Orla-Jensen, 1947). Few of the strains identified as *L. plantarum* were completely typical of that species. Heterofermentative strains represented a notable proportion of all the lactobacilli isolated. They were detected in the silage made at each of the three temperatures and at all stages in the fermentation. Of 159 strains of lactobacilli, to which a test for gas formation was applied, 56 were found to be heterofermentative. The majority of those examined in detail resembled most closely *L. fermenti* and *L. buchneri*. The strains attached to the latter species were atypical chiefly in failing to ferment melezitose.

In the present work a regular development of lactobacilli occurred solely in Expt. 10. Most of the silage made in Expt. 8 is believed to have been completely devoid of that group. The effects of temperature on the organisms seem to have depended in part on the species with which the grass happened to be seeded. Most of the strains of lactobacilli isolated in Expt. 9 were found to be capable of growth at 45°, and in that instance the best development occurred in the silage at 40°. The findings in Expt. 10 show that when the conditions in silage are favourable, the rate of multiplication of lactobacilli

at 30° can equal that of the other bacteria. In none of the experiments did lactobacilli show a definite competitive superiority during the phase of active growth. When the populations were declining lactobacilli retained their viability longer than most of the other organisms, although not as decisively as might have been anticipated. In Expt. 15, the silage at 30° after 6 months gave approximately equal counts of pediococci and lactobacilli.

Remainder. This fraction shown in Table 3 was obtained by subtracting the sum of the Gram-negative bacteria and lactobacilli from the count on glucose yeast agar, the latter being taken as approximating to a count of all bacteria other than the anaerobes. Organisms which contributed to the figures are those for which selective methods of culture were not used. *Streptococcus*, *Leuconostoc*, *Pediococcus* and *Bacillus* are the groups which showed multiplication to a significant extent. Other groups did occur in the silage. For example, low numbers of micrococci and yeasts were occasionally detected. Where the Remainder consisted largely of *Bacillus* (shown by B in Table 3) the figure gives a valid indication of the occurrence of that group. As estimates of the cocci the figures must be regarded as unreliable in those cases in which the Gram-negative organisms or the lactobacilli had reached the phase of decline in silage made at 22° or 30°, and also where these organisms were still giving some evidence of growth in the silage at 40°. In such circumstances the count of either group could be higher on glucose yeast agar than on the selective medium on which the group was enumerated, thus giving rise to a fictitious remainder. A similar variation in requirements for growth, associated with exposure of bacteria to adverse conditions, has frequently been recorded in the literature.

A large proportion of the streptococci appeared to be *Streptococcus faecium* Orla-Jensen or closely related organism. *S. faecalis* Andrewes & Horder and its varieties were isolated less frequently. *Leuconostoc* and *Pediococcus* formed well-developed colonies on acetate agar, but the validity of the counts so obtained is unknown. Acetate agar has not yet been tested for capacity to give maximum viable counts of these groups. *Leuconostoc* gave little evidence of growth at 40°, but multiplied actively in the silage at the lower temperatures. The other coccal forms developed at all three temperatures.

Bacillus was inactive at 22°; at 40° it formed a larger proportion of the whole bacterial population than it did at 30°. In Expts. 8 and 15 *Bacillus licheniformis* became the dominant organism at 40°. Its development in these cases resembled that shown by the Gram-negative rods in most of the silage made at 22°, thus suggesting that the two groups may be alternates which are selected by the ambient conditions. *B. coagulans* was demonstrated in all experiments at 40°. The counts of this organism showed little indication of decrease at the 8-day examinations, thus indicating that its powers of survival were similar to those of the non-sporing lactic acid bacteria. *B. polymyxa* multiplied in the two materials richest in nitrogen (Expts. 7 and 15). In these it was prominent after 6 months at 22° and 30°, an indication that it is capable of spore formation in silage.

Variations in the composition of the bacterial populations

The analyses of the populations given in Table 3 and discussed above provide information about the inter-group competition. They show that bacteria of all the main groups which were found to increase in the silage were capable of proliferation during the phase of general multiplication when the temperature was appropriate for the organism. The observation that *Lactobacillus* and *Clostridium* spp. may develop as early and as rapidly as other organisms demonstrates that fresh herbage deprived of oxygen provides conditions suitable for some of the more fastidious types of bacteria. There was a tendency when one of the bacterial groups attained dominance for the others to be correspondingly diminished, but at each temperature the relative proportions of the groups varied from one experiment to another as if they were due to differences in the inoculum or the conditions in the silage. Nilsson (1956) showed that on agar some silage bacteria can suppress the growth of others by means of antibiotic products. In the present experiments each bacterial group showed in one or other silage that it could multiply side-by-side with any other group, thus suggesting unimportance to antibiotics. Apart from the differential effects of temperature, few instances were detected of one group continuing to increase when the others were static or declining, which shows that inter-group competition during the multiplication phase was not strongly selective. This does not imply that in an unstable silage a renewed growth cannot occur during succeeding months. Evidence on the time of appearance of fermentation products obtained by Gneist (1937), van Beynum & Pette (1939), Irvin, Langston & Gordon (1956), Nilsson, Tóth & Rydin (1956), Rydin, Nilsson & Tóth (1956), and others, indicates that *Clostridium* and *Propionibacterium* spp. may initiate growth at a later stage if conditions are favourable; and Kroulik, Burkey, Gordon, Wiseman & Melin (1955) have shown a secondary increase of pediococci after several weeks. In the present work attention was purposely focused on the first week in view of some preliminary experiments which indicated that events in the early stages can have a dominating influence on the outcome of the whole fermentation.

Changes in pH value

The pH values of the grass and silage macerates are shown in Fig. 2. It will be seen that in several instances much acid must have been formed after bacteria of all the main groups had reached the phase of falling viable count. In each experiment except no. 10 the decreases in pH value were most rapid at 40° and slowest at 22°; the curves tended to approach each other with time; the final values were in no instance lower than 4.3. The silage in Expt. 10 differed in showing an active acid formation at the two lower temperatures, the curves for 30° and 40° being nearly identical; the final pH value at each of the three temperatures was lower than in the other experiments. Bacteriologically, the special features of the silage in Expt. 10 were the rapidity of the growth, the density of the populations attained, and the notable multiplication of

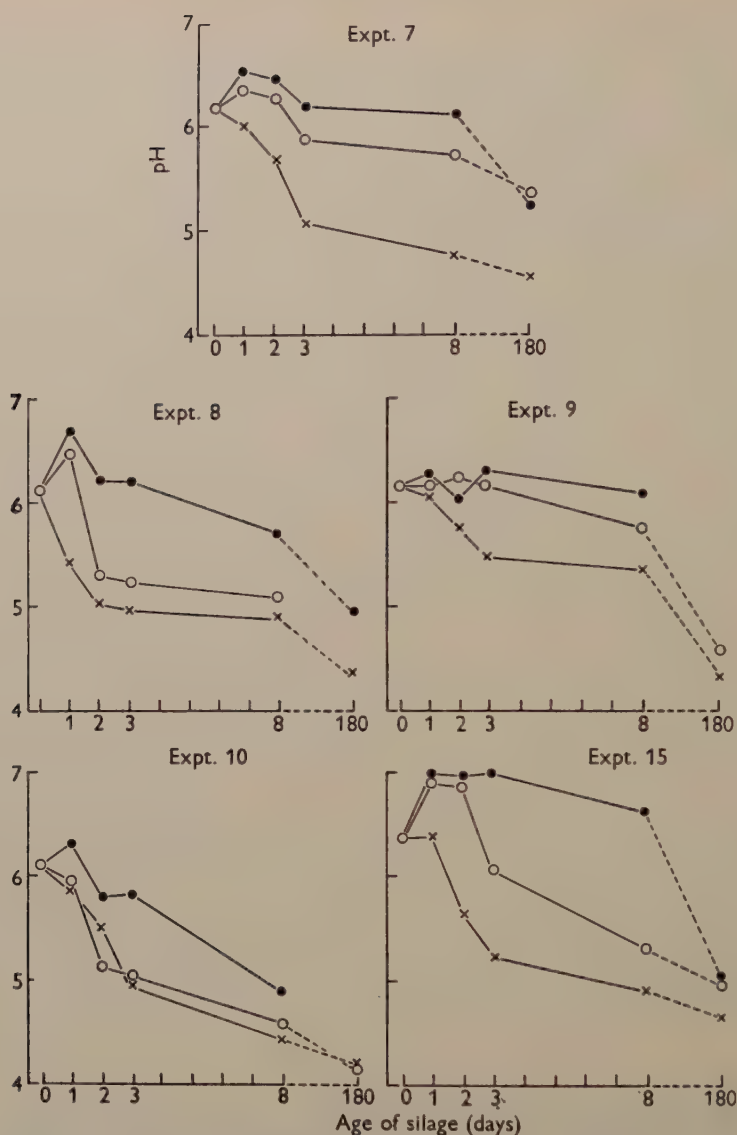


Fig. 2. The pH values of silage macerates. Silage made at 22°, ●—●; 30°, ○—○; 40°, ×—×.

lactobacilli. In view of the exceptional acid-producing powers of lactobacilli it seems likely that they were at least the immediate cause of the active acidification in this silage. It will be noted that in Expt. 9 the count of lactobacilli reached $220 \times 10^6/\text{g}$. Although apparently a substantial concentration of organisms, this figure is well below the maximum found in Expt. 10. It was evidently inadequate for rapid acid formation, at all events under the conditions provided by the grass in Expt. 9.

DISCUSSION

A feature of silage made from living herbage is its complexity. Diverse bacteria develop on plant materials which vary in character; the bacteria and the plant cells both produce chemical changes which are certain to have complex results. There are, consequently, obstacles to the identification of factors which determine the events. In the following discussion an attempt will be made to isolate some of the relationships. In the interests of clarity they will be discussed under five headings.

Factors which limit the bacterial growth. Acid formation has been recognized as a dominant factor in the control of bacterial activity in silage. The importance of acids is certainly well founded, yet in the present experiments growth was frequently arrested when, as the pH values show, little acid could have been formed. In the silage made from each specimen of grass it was generally found that the lower the temperature the higher was the pH value when the peak viable count of each bacterial group was obtained. It may therefore be concluded that the accumulation of acids was not the only factor responsible for the cessation of growth.

The simplest hypothesis is that bacterial multiplication was limited chiefly by the food supply. If this were the mechanism it would account for the finding that diverse types of bacteria stop growing at about the same time. Of the bacterial groups involved, the Gram-negative rods have the simplest nutrient requirements. It could therefore be inferred that in the specimens of herbage used, sugars, the chief sources of energy for anaerobic growth, were most frequently the limiting nutrilites. The finding in these experiments that the period of growth at 40° was similar to or longer than at 30° indicates that during the first week there was a more continuous supply of food material at the higher temperature.

The influence of temperature on the bacterial populations. The relation of the viable to the total bacterial counts in these experiments is unknown. In the light of the findings of Jordan & Jacobs (1947) it could be expected that the degree to which the viable counts underestimated the total counts was greatest for the silage at 40° and smallest for the silage at 22°, and that this would apply both during and after the phase of active growth. Whatever the limitations of the viable counts, some of the methods employed could prove the presence of various bacterial groups and hence give an indication of the extent of their development. The outstanding qualitative effects of the temperatures employed were the restraint of the Gram-negative organisms at 40° and of the spore formers, both the aerobic and the anaerobic, at 22°. Fig. 1, shows that the bacterial multiplication in the silage at 22° was remarkably uniform, apart from rate, in the five experiments. On the other hand, the viable counts and the composition of the populations in the silage at 40° showed much variation. The evidence obtained suggests that a differential effect of the higher temperature, operating on somewhat diverse populations, may explain much of the variability at 40°.

Herbage as a source of silage organisms. The strict aerobes, which were a

vast majority of the bacteria on the fresh grass, quickly perished after the laboratory silos were closed. In farm silage these organisms would have more opportunities of securing oxygen, yet they could not be expected to have much action in properly made silage. Their total respiratory activity and heat production are probably negligible in comparison with those of the much greater mass of plant material.

Bacteria capable of growth in anaerobic silage were shown to vary in rate of occurrence on the specimens of fresh grass. Their subsequent development in the silage provided a strong indication that the representation of a bacterial group on the herbage is one of the factors which determine the success of that group in competition with others after ensilage. Evidence of this relationship was given more especially by the lactobacilli, a group which could be enumerated when heavily outnumbered. Only one specimen of grass, that used in Expt. 10, carried more than a very thin seeding with these organisms. The count of *Lactobacillus* on that grass, although it amounted to only about 1 in 20,000 of the total herbage bacteria, was unusually high as is shown by Stirling's (1953) results. It was associated with an exceptionally early and extensive multiplication of lactobacilli in the silage. These observations suggest that the relationship of the original seeding with lactobacilli to their growth in silage is one that deserves further exploration.

The examinations of the silage gave indirectly some information about the occurrence on grass of certain relevant organisms. Thus, the results of Expt. 8 make it virtually certain that lactobacilli were absent in that instance from several of the 50 g. quantities of the grass ensiled. The work on pure cultures of *Lactobacillus* showed (1) that the species which multiplied in the silage varied from one experiment to another, and (2) that in every experiment, including no. 8, two or more species developed. The clostridia showed similar variations although some species possibly occurred more constantly. All this evidence leads to the conclusion that irregular chance contamination plays a large part in determining the occurrence on herbage of the typical silage bacteria. A high count presumably signifies that the organisms had proliferated on the aerial parts of the growing plant.

The influence of the properties of the herbage. In the silage made from the five specimens of grass the bacterial development varied in rate and in the concentration of viable organisms reached. The rapid and extensive growth in Expt. 10 contrasts with the delayed and restricted multiplication in Expt. 15. The variations do not show a simple relationship to the types of bacteria that were found to have multiplied. It may therefore be concluded that the specimens of herbage differed in their suitability for supporting bacterial growth. In the early stages of the silage fermentation the bacterial proliferation is believed to occur only on the plant surfaces (Ruschmann, 1939). If that be true, juice derived from the plant tissues is the medium in which growth will occur. The rate at which it is liberated and its composition are known to vary, and could be presumed to be the factors responsible for the differences in bacterial activity.

The rate of change in pH value also differed from one experiment to another.

The presence of specific organisms does not appear to account for all the differences. Neither does a variation in the initial buffering capacity of the grass since the pH values after 6 months do not correspond with the earlier rates. An explanation which suggests itself is that acid formation is limited by the supply of sugars at the sites of bacterial growth. On this view, in Expt. 9 the feeble acid production up to the eighth day and the considerable fall in pH value by 6 months might be attributed to a slow release of fermentable sugars. That the concentration of sugar in herbage is often insufficient to permit an adequate acidification of silage is well established. In none of the experiments here described does sugar appear to have been severely deficient, although the pH curves suggest that it was completely utilized in some cases. No example was included of the type of grass that yields a markedly unstable silage in which the sugars are totally consumed, the acids are destroyed and the material rots.

The accumulation of acid in relation to temperature. With the exception of Expt. 10, where lactobacilli appear to have had a dominating influence, the pH data show no simple relation to the bacteriological findings. If decreases in the pH value of silage are due to the production of fermentation acids, and the available evidence indicates that this is so, it seems necessary to assume that during the first week acids were formed by bacteria which had ceased to grow or had lost their viability. Investigations of lactic acid bacteria in other media, for example those of Rogers & Whittier (1928), Longworth & MacInnes (1936) and Stern & Frazier (1941), have shown that there are various conditions which arrest growth but permit fermentation to continue. Silage would not be exceptional if it provided this situation. The special feature of silage, in contrast with pure cultures in media such as broths or milk, is in the relationship of temperature to acid formation shown by the curves of Fig. 2. A pattern of the lowest pH values at 40° and the highest at 22° is sufficiently distinctive to indicate that it is characteristic of silage in which the lactic acid fermentation is not vigorous. The effects of temperature on the bacteria and on the herbage are doubtless complex. One possibility, which does not appear to have been explored, is that the rate of sugar liberation from the plant tissues may increase with rising temperature. The decreases in pH value between 8 days and 6 months, and the tendency for the curves to converge during this period, suggest that sugars became available for fermentation, and that there was finally some compensation for a slow acid formation in the early stages. Sugars formed by the breakdown of polysaccharides (Wylam, 1953; Harwood, 1954; de Man 1957) might be expected to have contributed to the final results. The present work did not show whether a renewed growth of lactic acid bacteria occurred in the period between eight days and six months.

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A Quantitative Study of the Production of Dextran from Sucrose by Rumen Strains of *Streptococcus bovis*

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SUMMARY: Freshly isolated and old stock strains of *Streptococcus bovis* originating from the rumen will produce dextran at 37° in liquid sucrose-containing media. For good yields the presence of CO₂ in some form is necessary. The CO₂ may be provided as HCO₃⁻ at the start or during the life of the culture or by incubation in a CO₂ atmosphere. The dextran has $[\alpha]_D^{20} + 187^\circ$ to $+190^\circ$ and is similar chemically to the leuconostoc dextran save that branching of the $\alpha 1 \rightarrow 6$ linked anhydro-glucose chain is rarer. With some strains practically no dextran is formed in H₂ as gas-phase or in a closed system without HCO₃⁻ from which air is excluded. Other strains seem to have a limited power of producing dextran under these conditions, possibly because their action is not entirely homofermentative. Tween 80 will partially replace CO₂ even with the first kind of strain. Highest yields of dextran, up to 80 % of the anhydro-glucose provided, are obtained when the life of the culture is prolonged by repeated neutralization and when additional sucrose is supplied. This is best achieved by the continuous neutralization obtained when solid CaCO₃ is present in the culture. Dextran production is always accompanied by accumulation of fructose in the culture liquid together with a reducing fructose-containing disaccharide. Dextran can sometimes be produced in a simple liquid sucrose + proteose peptone medium with no phosphate buffering. Other things being equal, the presence of CO₂ or HCO₃⁻ does not greatly increase the yield of bacterial protein in sucrose media.

Streptococcus bovis or a similar amylolytic group D streptococcus is always present in the rumen (see MacPherson, 1953; Hungate, 1957). Strains of this organism have been reported to produce at least two types of polysaccharide: a capsular polysaccharide and a water-soluble polyglucosan. The capsular polysaccharide contains galactose, rhamnose and uronic acid units (Hobson & MacPherson, 1954) and is produced in media which contain glucose or presumably any other fermentable carbohydrate. There is no requirement of CO₂ for its formation and in older cultures it may also be found free in solution although admittedly in very low concentrations. As the capsular compound does not contain glucose there should be no difficulty in determining which of the two types of polysaccharide is responsible for the sliminess of any given *S. bovis* culture.

Hehre & Neill (1946) showed, chiefly by serological tests, that many strains of 'viridans' streptococci isolated from the blood of patients with subacute bacterial endocarditis could convert sucrose into a polysaccharide indistinguishable from leuconostoc dextran. *Streptococcus bovis* is in many respects also a 'viridans' streptococcus (Topley & Wilson, 1946). Niven, Smiley & Sherman (1941) originally stated that several strains of *S. bovis*, of unrecorded origin, produced from sucrose a soluble polysaccharide which was described as 'dextran' because it yielded glucose on hydrolysis. More recently, Dain,

Neal & Seeley (1956) reported that the generality of *S. bovis* strains, including those isolated from cattle rumen, formed large glucosan-containing mucoid colonies (as distinct from the usual non-mucoid pinhead 'typically streptococcal' colonies) on nutrient agar only when provided with sucrose and a carbon dioxide atmosphere. The polysaccharide was shown to be a polyglucosan but the major linkage and hence the polysaccharide was not identified. In corresponding liquid cultures they stated that 'the cells tended to remain in suspension even after several days and viscosity of the medium was increased although in no case did complete solidification occur.' Hence these authors did not show that the organism is capable of producing the polyglucosan in liquid media nor that the polyglucosan produced on agar is dextran. In contrast to the above work Dr J. C. Appleby, formerly of the Rowett Research Institute, informed us that in 1956 she isolated from two sheep rumens a strain of *S. bovis* which produced much polyglucosan on sucrose nutrient agar in a hydrogen atmosphere. There was apparently no stimulation of polyglucosan production by CO₂. It seems likely therefore that such strains are not uncommon.

Most of the work on dextrans has hitherto been done on material produced by *Leuconostoc* spp. Following these investigations dextrans may be defined as water-soluble polyglucosans in which the major linkage is the $\alpha 1 \rightarrow 6$ glucosidic link (see Barker *et al.* 1955). Varying proportions (0–20 %) of $\alpha 1 \rightarrow 3$ and $\alpha 1 \rightarrow 4$ glucosidic linkages have been reported to be present in the dextrans produced by different *Leuconostoc* spp. (Jeanes & Wilham, 1952; Barker *et al.* 1954). These secondary linkages are believed to occur as branch points along the main chain of $\alpha 1 \rightarrow 6$ linked glucose units. The presence of the $\alpha 1 \rightarrow 6$ glucosidic link, as the major linkage, must be demonstrated to show that any polyglucosan is in fact dextran.

Leuconostoc spp., which, unlike *Streptococcus bovis*, are heterofermentative (i.e. CO₂ producers) require neither added CO₂ in the gas phase nor HCO₃⁻ to give good dextran yields from sucrose-containing liquid media. It seemed therefore desirable to establish whether CO₂, in some form, was necessary for dextran production by *S. bovis*, particularly in liquid media. It also appeared necessary to show clearly to what extent the polysaccharide resembled true leuconostoc dextran. Such results are of practical interest since in the rumen *S. bovis* lives in a bicarbonate buffer in the presence of a gas-phase containing CO₂. When the animal consumes a sucrose-containing ration such as spring grass or clover the organism may produce a dextran slime. Hungate, Fletcher, Dougherty & Barrentine (1955) in fact believed that slime production in the rumen 'may be associated in an important way with bloat in ruminants'.

In this paper we show that dextran, produced from sucrose only, is responsible for the greatly increased viscosity of suitable liquid cultures of rumen strains (old and freshly isolated) of *Streptococcus bovis*. There is an absolute requirement for CO₂ to give appreciable dextran production by most, but not all, strains of this organism no matter how highly the medium may be buffered by phosphates, etc. We also record preliminary observations on the structure of the *S. bovis* dextran and on the nature of the soluble sugars found

in high dextran-producing cultures. An account of the properties of *S. bovis* dextransucrase is reserved for a subsequent publication. For the purpose of the present paper it has been considered sufficient to show that the polymer is a polyglucosan and that the major linkage present is the $\alpha 1 \rightarrow 6$ glucosidic linkage. This has been done by the chromatographic identification of isomaltose in partial hydrolysates and glucose in total hydrolysates of the dextran. Further confirmation has been obtained by periodate oxidation and by measurements of $[\alpha]_D$ values.

METHODS

Strains of rumen Streptococcus bovis and of Leuconostoc mesenteroides

Old stock cultures. Seven strains of *Streptococcus bovis* were obtained, freeze dried, from the Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland. They had been isolated from the rumen of sheep and calves in work described by Macpherson (1953); Mann, Masson & Oxford (1954); Mann & Oxford (1955). Their reference numbers are 18/C2, 18/M2, 19/C1, 2B, 16B, 2SA, and 1010 Y.

Freshly isolated strains of Streptococcus bovis. These were isolated from muslin-strained rumen liquor obtained from three fistulated cows feeding on rye grass or red clover at this laboratory. Two isolation procedures were used.

(1) A loopful of a 1/2500 dilution of rumen liquor in sterile 0.5% (w/v) yeast extract (Difco) was inoculated into liquid 2% (w/v) sucrose Bactiothioglycollate medium (10 ml., Difco; containing 0.075% (w/v) agar but no glucose) and incubated for 1 day in air at 37°. A loopful of the shaken culture was streaked on several plates of 3% (w/v) sucrose + 3% (w/v) proteose peptone no. 3 (Difco) agar and the plates incubated at 37° for 1 day in CO₂ (see below). The first plate invariably showed confluent mucoid growth but single well-separated mucoid colonies were always found on the other plates. No other organisms were ever encountered under these conditions.

(2) Crude rumen liquor was centrifuged for 30 min. at 40,000 g, and 0°. The supernatant fluid was discarded. One small loopful (0.001 ml.) of the yellow slimy layer on the surface of the centrifuged solid was inoculated into the sucrose + thioglycollate liquid medium (10 ml., above). Two successive serial dilutions were prepared by transferring 2 large loopfuls (0.02 ml.) through 2 samples (10 ml.) of sucrose + thioglycollate liquid medium containing 0.075% (w/v) agar. After incubation at 37° with CO₂ as gas phase the final dilution almost invariably showed a few discrete growths of *Streptococcus bovis* which were purified by plating as above. Plating on the sucrose + proteose peptone agar in CO₂ always produced mucoid colonies whereas with sucrose + Brewer's anaerobic agar (Difco, no glucose or methylene blue) the colonies were often non-mucoid. No explanation is offered for this fact, and buffering the medium with phosphate did not alter it.

Nine new strains of *Streptococcus bovis* were thus isolated, denoted I (cow I); A1, A2, 293C, 30 and 34 (cow 293); and IV, 40 and 44 (cow 294). Strains I, A1, A2, and IV were obtained when the cows were on a diet of pure rye grass (*Lolium* species) and the other strains when they were on a diet of pure red

clover (*Trifolium pratense*). For the main quantitative experiments *S. bovis* strains 1010Y (old) and I (new) were used. All cultures were maintained in Difco tomato juice agar or in 0.25% sucrose + thioglycollate (Difco) agar slabs kept at 0°.

Leuconostoc strains. For comparative purposes a culture of *Leuconostoc mesenteroides* (NCIB 8590) was obtained from the National Collection of Industrial Bacteria (D.S.I.R.), Teddington, Middlesex, England.

Liquid media

For dextran production. Cultures of *Streptococcus bovis* in 3% (w/v) sucrose + 3% (w/v) proteose-peptone no. 3 (Difco) liquid medium without agar did not always yield dextran in a CO₂ atmosphere; an exception with *S. bovis* strain IV is described below. The following modified Bacto-thioglycollate liquid medium was invariably successful. Its composition at three concentrations ($a < b < c$) of phosphate buffer was (% w/v): Bacto-yeast extract, 0.5; Bactocastone, 1.1, L-cystine (dissolved separately in a little boiling 0.1N-HCl), 0.025; sodium thioglycollate, 0.03; K₂HPO₄, (a) 0.45; (b) 0.54; Na₂HPO₄ 12H₂O, (c) 1.2; KH₂PO₄, (a) 0.15; (b) 0.18; (c) 0.20; (NH₄)₂HPO₄, 0.03; sucrose, usually 8; pH 7.0. All salts (except K₂HPO₄, British Drug Houses Ltd) were of Analar quality.

For an initial pH value between 7.0 and 8.0, ammonia solution (sp.gr. 0.91) was added, drop by drop, to give the desired value. Sucrose could safely be sterilized in solution in this basal medium; other sugars to replace sucrose were sterilized separately in concentrated aqueous solution. Basal medium free from CO₂ and HCO₃⁻ was obtained sterile by three successive steamings, not by autoclaving. In this case the medium nearly filled a conical flask (1 l.) carrying a rubber bung pierced with a piece of open glass tubing. After each steaming the flask was removed, cooled rapidly, and the outlet tube closed. After the final steaming the medium was rapidly dispensed into small sterile conical flasks (50 or 100 ml.) containing any required sterile supplements (see below), inoculated, and plugged. The inoculum was 1 loopful/50 ml. medium, from an 18 hr. culture at 37° in liquid 2% (w/v) sucrose + Bacto-thioglycollate medium.

Capsulation of the bacteria. The presence of capsules was demonstrated by negative staining, with 0.25% (w/v) nigrosine, of the washed bacteria obtained by centrifuging cultures (5 ml.) at 15,000 g.

Cultural techniques

Incubation techniques were designed to cover: (a) growth in the absence of CO₂ or HCO₃⁻ but in the presence of oxygen or hydrogen; (b) anaerobic growth in the presence of controlled amounts of HCO₃⁻; (c) growth with a CO₂ gas phase, with or without control of pH value; (d) growth in the presence of CaCO₃ giving continuous CO₂ and pH control. All cultures were incubated at 37° for a specified time. This incubation temperature was below that of the rumen (40°). *Leuconostoc dextran* sucrose is rapidly destroyed at 40° (Koepsell & Tsuchiya, 1952) and it was therefore decided to incubate the

Streptococcus bovis strains a little below this temperature. In cultures incubated for more than 1 day where it was judged that good dextran production would occur, more sucrose was added every 24 hr. as a sterile solution (4 ml. of 25 % (w/v) solution/50 ml. of culture).

Growth in the absence of CO₂ or HCO₃⁻. Conical flasks (50 ml.) nearly filled with media were used. They were either closed with a rubber bung or fitted with a bung carrying an open tube filled with self-indicating soda-lime. Cultures were also grown in shallow layers of medium in cottonwool-plugged flasks. Growth under a hydrogen gas phase was done in cottonwool-plugged flasks within a metal anaerobic jar (see below). Tween 80 (Atlas Powder Co. N.Z.) at 1 % (v/v) was added to several of these cultures.

Growth in the presence of controlled amounts of HCO₃⁻. The cultures were grown in the conical flasks closed with rubber bungs. Before inoculation the calculated amount of heat-sterilized K₂CO₃ solution (5.4 % w/v) was added.

Growth in a CO₂ atmosphere. This was carried out by placing the inoculated cultures, in cottonwool-plugged flasks, in a 4 l. metal anaerobic jar (Messrs Gallenkamp Ltd). Not more than 1 l. of liquid was incubated at a time so that the weight of CO₂ in the jar (> 6 g.) was sufficient, if all had dissolved in the liquid medium during incubation, to give a concentration at least 0.1 M in the medium. Air was displaced by a stream of water-washed CO₂, from a cylinder, for at least 30 min. No special effort was made to free the CO₂ from traces of oxygen.

Growth in the presence of CaCO₃. The sterile medium was added to sterile finely divided CaCO₃ (3 g./50 ml.) just before inoculation and the flask loosely plugged with cottonwool.

Control of the pH value of the cultures

The pH value of the growing cultures fell from 7.0–7.5 to 4.0–4.5 in 24 hr. When required the pH value was adjusted, with sterile aqueous ammonia (sp.gr. 0.95), to 7.0 (or 6.7 if the buffering capacity was high) every 24 hr. This was done by the cautious addition of ammonia whilst checking the pH value with narrow range indicator papers (British Drug Houses Ltd) with aseptic precautions. The ammonia solution was previously sterilized by heating it to the boiling point, rapidly cooling, and repeating the process 3–4 times during 30 min. Inoculation of this solution into sterile nutrient broth never showed growth when incubated at 37° for 3 days. When it was desired to introduce CO₂ or HCO₃⁻ into the growing culture sterile K₂CO₃ (20 %, w/v) was used to adjust the pH value.

Dextran estimation

Qualitative detection. Soluble polysaccharide was detected by the addition of ethanol (4 vol.) to the supernatant fluid obtained after centrifuging the culture (5 ml.) for 30 min. at 13,000 g. When dextran was present the solution yielded a heavy precipitate or a turbidity which readily flocculated after shaking for a few minutes. Cultures containing dextran were always opalescent. Uninoculated medium or clear culture fluid containing no dextran

yielded, on adding ethanol, a turbidity which did not flocculate even after prolonged shaking.

Quantitative measurement of dextran. A volume (50 ml.) of culture was centrifuged for 30 min. at 13,000 g to remove bacteria. Very viscous cultures were diluted with an equal volume of water before centrifuging, whilst cultures containing CaCO_3 were acidified with $2\text{N-H}_2\text{SO}_4$ to remove calcium and to avoid the precipitation of calcium lactate by the ethanol. Dextran was precipitated from the centrifuged fluid by the slow addition of ethanol (2 vol.) and then standing the mixture at 0° overnight. The clear supernatant fluid was decanted and kept for paper chromatographic analysis. After draining, the precipitate was dissolved in water (100 ml.), boiled for 3 min., recentrifuged, and reprecipitated with ethanol (2 vol.). The final precipitate was dissolved in water, centrifuged, and made up to volume (100 or 250 ml.) for analysis. After diluting a portion of the solution (1–5 ml.) to 100 ml. with water, dextran was determined by the anthrone method of Roe (1954), a carefully purified sample of the streptococcus dextran being used as a standard. The extinction value of the developed colour was measured, at 625μ , on a Beckman model DU spectrophotometer with 1 cm. thick optical cells. The yield of dextran was expressed as % conversion of the available anhydroglucose of the sucrose to polymerized anhydroglucose (i.e. dextran). The medium itself, when submitted to the above analysis, gave no measurable amount of polysaccharide.

Yield of bacteria

The solid residue obtained from the first centrifugation of the culture (50 ml.) was suspended in water (70 ml.), thoroughly shaken and centrifuged for 30 min. at 13,000 g. The washed organisms were freeze-dried, weighed, and analysed for total-N by the micro-Kjeldahl method.

Structural investigations on the dextran

Purification of the dextran. Dextran solution obtained in the analytical procedure (above), was further purified to remove traces of associated protein. This was achieved by shaking the solution four times with an equal volume of chloroform + amyl alcohol (2.5:1, v/v) and centrifuging to remove the emulsion formed (Sevag, Lackman & Smolens, 1938). The purified dextran was precipitated with ethanol (2 vol.), dissolved in water, centrifuged, dialysed overnight, freeze dried and finally dried under vacuum at 60° .

Hydrolysis of the dextran. Dextran (20 mg.) dissolved in $1.5\text{N-H}_2\text{SO}_4$ (10 ml.) was heated for 9 hr. at 100° , neutralized (3N-NaOH) and diluted to volume (100 ml.). Reducing sugars were measured in the hydrolysate, as glucose, by the method of Shaffer & Hartmann (1921). The results obtained were corrected by the factor given by Pirt & Whelan (1951) for these conditions. For paper chromatographic analyses the hydrolysate was neutralized with solid BaCO_3 , filtered, and concentrated to 0.2 ml. Partial hydrolyses were carried out by heating as above for 1.5 hr.

Paper chromatographic analyses. These were prepared from the hydrolysates

and ethanol supernatants, by the usual techniques and developed with either of the following solvents:

(a) The top layer of a mixture consisting of *n*-butanol, ethanol, water and ammonia (49, 10, 40 and 1 %, v/v; Barker *et al.* 1954).

(b) The top layer of a mixture containing ethyl acetate, water and pyridine (2:2:1 v/v; Jermyn & Isherwood, 1949). Sugars were detected with the following sprays: silver nitrate (Trevelyan, Proctor & Harrison, 1950), aniline hydrogen phthalate (Partridge, 1949), naphthoresorcinol (Partridge, 1948), β -indolylacetic acid (Heyrovsky, 1956), benzylamine + ninhydrin (Bayly & Bourne, 1953).

Periodate oxidation and optical rotation of the dextran

Dextran (100 mg.) was oxidized and periodate consumption and formic acid production measured by the methods referred to by Bailey, Barker, Bourne & Stacey (1957). Optical rotations were measured on solutions of dextran (30 mg.) dissolved in *N*-NaOH (10 ml.) with a Hilger polarimeter and a 1 dm. tube. Because of the opalescence of dextran solutions exact readings were difficult to obtain.

RESULTS

Fermentation and other reactions of the Streptococcus bovis strains

All 16 strains of *Streptococcus bovis* were catalase-negative, morphologically similar, Gram-positive cocci and were typical *S. bovis* in the sense of Mann & Oxford (1955) in that all were amylolytic, and fermented raffinose and inulin but not mannitol. Only one strain (293C) fermented arabinose, causing a fall in pH to 4.5. No strain produced dextran, in the presence of CO₂, from any simple sugar or oligosaccharide save sucrose. Fructose was fermented by strain I at least. All strains produced very similar mucoid colonies on 3 % (w/v) sucrose + 3 % (w/v) proteose peptone No. 3 (Difco) agar on incubation for 1 day in a CO₂ atmosphere at 37° or 40°. All were homofermentative towards glucose even in the presence of Tween 80 (1–3 %) and brought about a decrease of pH to 4 or less in 1 day at 37°.

Capsulation of the bacteria. All of the strains of *Streptococcus bovis*, particularly in 3-day cultures with pH control, produced zoogloeal masses of capsulated cocci in all of the cultures including those producing dextran. *Leuconostoc mesenteroides* also produced capsulated organisms, indistinguishable from those of *S. bovis*, in sucrose-containing cultures.

Qualitative observations on the need for CO₂ to obtain good dextran production in liquid media

Streptococcus bovis. All strains gave obvious dextran production in the 8 % (w/v) sucrose + phosphates liquid medium (cf. p. 133) when incubated for 1 day in a CO₂ atmosphere. It seemed to make little difference in this respect whether the surface/volume ratio of the liquid culture was large or small. The cultures became viscous and opalescent and a clean supernatant fluid could not easily be obtained by centrifuging. Another way of providing CO₂ with-

out using a CO_2 atmosphere was to neutralize with K_2CO_3 after some growth had taken place with a consequent fall in pH.

The preliminary results are summarized in Table 1 from which it will be seen that yields of dextran of over 50% of the theoretical were obtained and that phosphate buffering could be dispensed with (see strain IV, Table 1); presumably ammonium lactate acted as the buffer in the sucrose + proteose-peptone medium after some growth had taken place.

Table 1. *Yields of dextran produced by various strains of Streptococcus bovis, in liquid media, in the presence of CO_2*

All media contained 8% (w/v) sucrose unless otherwise stated. Incubation was for 3 days at 37° . See p. 133 for composition of the medium.

<i>S. bovis</i> strain	Medium	Volume (ml.)	Cultural conditions	Dextran yield; g. (% conversion of available anhydro- glucose)
A ₁	Phosphate (a)	100	CO_2 atm.; no neutralization	0.36 (9.49)
A ₁	Phosphate (b)	100	CO_2 atm.; neutralized with K_2CO_3 after 1 day	0.68 (17.94)
A ₂	Phosphate (a)	100	CO_2 atm.; no neutralization	2.55 (67.28)
A ₂	Phosphate (a)	100	No CO_2 atm.; neutralized with K_2CO_3 after 1 and 2 days	1.75 (46.17)
18/M2	Phosphate (b)	75	CO_2 atm.; neutralized with K_2CO_3 after 1 day	2.12 (83.46)
293C	Phosphate (c)	65	CO_2 atm.; no neutralization	0.67 (27.23)
I	Phosphate (b)	75	CO_2 atm.; no neutralization	1.29 (50.78)
1010Y	Phosphate (a)	100	CO_2 atm.; neutralized with K_2CO_3 after 1 and 2 days	1.44 (37.99)
IV	3% sucrose + 3% (w/v) pro- teose-peptone No. 3 (no phosphate)	200	2 days CO_2 atm.; neutralized with NH_4OH and more suc- rose (8.5 g.) added after 2 days; 3rd day in air	4.00 (50.16)

In the complete absence of CO_2 , either in the closed full flask or in air under soda-lime, the results were not so clear cut. Some strains of *Streptococcus bovis*, e.g. I, 18/M2 consistently gave practically no dextran in 3 days (see, however, Table 3 for the effect of Tween 80 in substitution for CO_2 with strain I), even though obvious growth had occurred in 6 hr. Other strains sometimes gave a good dextran production in the absence of CO_2 , even in a closed system with oxygen excluded, e.g. strains 293C, 34, 40. The results were not consistent, strain 293C for example giving no dextran in a closed system on another occasion. In every instance, however, the presence of CO_2 either in the gas phase or in solution at 0.10 M did increase the yield of dextran. Shallow layer cultures in a CO_2 -free medium, incubated in air, invariably produced some dextran. All *S. bovis* strains can probably utilize oxygen to some small extent since even freshly isolated strains are facultative anaerobes. It was noted that cultures, even in nearly full flasks under soda-lime sometimes produced enough CO_2 to change the colour of the bottom few mm. of the soda-lime layer.

Leuconostoc mesenteroides (NCIB 8590). This organism differs from *Streptococcus bovis* in being markedly heterofermentative. In our hands it gave a quicker growth and dextran production from 4% (w/v) sucrose medium, with half the phosphate buffering of (a) (p. 133) at 29° in either a closed system or CO₂-free air, than in air or in a CO₂ atmosphere not freed from oxygen. This may merely mean that the particular strain used was microaerophilic. The results were similar in all instances after 2–3 days of incubation. Gas production in the closed system cultures was very obvious.

Minimum concentration of HCO₃⁻ required for good dextran production with Streptococcus bovis strain I incubated in a closed system

The results summarized in Table 2 indicate that, with *Streptococcus bovis*, strain I, an initial CO₂ concentration of at least 0.005M (as HCO₃⁻) was necessary for good dextran production when neither CO₂ gas nor oxygen was provided. Results from a similar experiment with *S. bovis* strain 1010 Y were much

Table 2. *Effect of HCO₃⁻ concentration on the yields of dextran produced by Streptococcus bovis, strain I*

Medium (c) p. 133; 50 ml. (containing 4 g. sucrose) in a closed system; incubated for 2 days at 37°.

Initial K ₂ CO ₃ (M)	Yield of organism		Dextran as % conversion of available anhydro- glucose	Sugars in supernatant fluid at end		
	Dry wt. (mg.)	Protein (N × 6.25) (mg.)		Fructose	Sucrose	Reducing disac- charide
None	58	29.00	4.76	—	++++	—
0.0004	71	35.50	8.73	+	++++	—
0.0017	89	41.00	16.40	++	++++	+
0.005	87	46.00	39.15	+++	+++	+
0.014	90	42.10	50.27	++++	++	++
0.028	No growth		—	—	—	—

more erratic. This is one of the strains that does not seem invariably to need CO₂ in some form for dextran production. Neither strain grew in phosphate medium (c) in the presence of 0.028M-HCO₃⁻, even though the initial pH was below 8; presumably the salt concentration was too high.

It was obviously necessary to show that the extra buffering power of the medium due to the addition of K₂CO₃ was not the cause of dextran production. Medium (c) was therefore made a stronger phosphate buffer by the addition of Na₂HPO₄, 12H₂O (0.3%; 0.008M) and KH₂PO₄ (0.05%; 0.004M) without change of pH value. Parallel cultures of *Streptococcus bovis* strain I in medium (c) modified and unmodified (each neutralized with ammonia after 1 and 2 days) in the absence of CO₂ in a closed system gave no qualitative reaction for dextran in either instance although growth was profuse. When the third day of incubation was in a CO₂ atmosphere, however, there was good dextran production in each instance.

*Minimum sucrose concentration necessary for dextran production
in a CO₂ atmosphere*

Five strains of *Streptococcus bovis* (A1, I, 293C, 1010Y, IV) all behaved similarly in that no dextran was detected qualitatively in liquid thioglycollate medium (c) cultures containing initially 0.25% (w/v) sucrose (final pH 6.1). The polysaccharide was, however, definitely produced from 0.5 (w/v) sucrose medium (final pH 5.5–5.7). The final pH value was always below 5 when the initial sucrose concentration exceeded 0.75%.

The effect of various cultural conditions on growth and dextran production

The results obtained, with *Streptococcus bovis* strains 1010Y and I are detailed in Table 3. It is evident that the presence of CO₂ increased dextran yield, with or without pH control, although it seems to have had little effect on yields of organism. Maintaining the pH at 6.5–7 improved dextran yields in CO₂ and gave an increased yield of organism. The best way so far found for fulfilling the above conditions, i.e. addition of CaCO₃, gave the highest yields (nearly 80% conversion of available anhydro-glucose with strain I) obtained for each strain. The results however (Tables 1 and 3) do show considerable strain differences in the effect of pH control and CO₂ on dextran production. Tween 80 did replace CO₂ to some extent in stimulating dextran formation. Un-neutralized cultures incubated in CO₂ for 1 or 3 days gave closely similar yields of organism and dextran. These results emphasize the need for pH control, as well as for CO₂ in order to obtain optimum dextran production and cell multiplication.

Chromatographic examination of the cell-free supernatant fluid showed that, as with *Leuconostoc mesenteroides* NCIB 8590, fructose appeared concurrently with dextran formation. The occurrence of other oligosaccharides in the cultures is discussed below.

Structure of the dextran

Structural studies were confined to the dextrans produced by the two strains of *Streptococcus bovis*, 1010Y and I, used in the experiments described in Tables 2 and 3 and isolated from the cultures described in Table 1. Chromatograms of total hydrolysates showed a single reducing component chromatographically identical with glucose. There was no sign of any of the component sugars (galactose and rhamnose) of the capsular polysaccharide or of fructose. Chromatograms of partial hydrolysates, developed with solvent (a), showed a single reducing component, $R_{glucose}$: 0.40, chromatographically identical with *iso*-maltose produced by the partial hydrolysis of *Leuconostoc* (*Betacoccus*) *arabinosaceus* dextran (Barker *et al.* 1954): $R_{glucose}$: isomaltose 0.40; maltose 0.50; cellobiose 0.47.

When sprayed with aniline hydrogen phthalate the disaccharide component gave the same brownish yellow colour as isomaltose. The component was also identical with isomaltose on chromatograms developed with solvent (b) and by the benzylamine technique with solvent (a). In this latter case the

Table 3. *Yields of dextran, and bacterial protein, produced by Streptococcus bovis, strains I and 1010 Y under various cultural conditions*

Medium (c) p. 133 (50 ml. containing 4 g. sucrose, initially) in all cases; incubation for 2 days at 37° unless otherwise stated.

Gas phase	Neutralization during incubation	Supplements added after 1 and 2 days	Bacterial cell yields		Dextran as % conversion of available anhydro-glucose	Sugars in supernatant at end of growth			
			Dry wt. (mg.)	Protein (N × 6.25) (mg.)		Fructose	Glucose	Sucrose	Reducing disaccharide
Strain I									
None; closed system	None	None	48.0	22.4	7.14	0	0	+++	0
None; closed system	None	Tween 80 (1 % at start)	54.0	26.8	16.40	++	0	+++	+
Hydrogen	None	None	68.0	20.8	0	0	0	+++	0
CO ₂ -free air; (1 day)	None	None	36.7	21.2	6.5	+	0	+++	+
CO ₂ -free air; (3 days)	None	None	47.7	23.0	6.7	+	0	+++	0
CO ₂ -free air; (2 days)	NH ₄ OH (once)	Sucrose (1 g.)	92.3	36.4	16.4	++	0	+++	+
CO ₂ -free air; (3 days)	NH ₄ OH (twice)	Sucrose (1 g.)	171.0	67.6	18.6	+++	+++	++	++*
CO ₂ (1 day)	None	None	52.5	27.2	32.2	++	0	+++	+
CO ₂ (3 days)	None	None	66.1	22.2	34.7	+++	0	+++	+
CO ₂ (2 days)	NH ₄ OH (once)	Sucrose (1 g.)	102.3	48.3	50.0	+++	0	0	+++
CO ₂ (3 days)	NH ₄ OH (twice)	Sucrose (1+1 g.)	116.9	54.0	59.0	+++	0	0	+++
Air	CaCO ₃ continuously	Sucrose (1+1 g.)	—	—	79.2	++	0	0	+++
Strain 1010 Y									
None; closed system	None	None	—	—	9.0	+	0	+++	0
None; closed system	NH ₄ OH (once)	Sucrose (1 g.)	64.5	32.9	7.1	+	0	+++	0
Hydrogen	None	None	74.9	34.0	1.5	0	0	+++	0
CO ₂ -free air; (1 day)	None	None	47.6	27.6	20.2	+++	0	+++	0
CO ₂ -free air; (3 days)	None	None	52.8	25.6	19.0	+++	0	+++	+
CO ₂ -free air; (2 days)	NH ₄ OH (once)	Sucrose (1 g.)	30.3	16.4	11.6	++	0	+++	+
CO ₂ -free air; (3 days)	NH ₄ OH (twice)	Sucrose (1+1 g.)	137.0	50.5	38.3	+++	++	++	++*
CO ₂ (1 day)	None	None	45.0	23.4	29.4	+++	0	+++	+
CO ₂ (3 days)	None	None	50.2	26.4	30.3	+++	0	+++	+
CO ₂ (2 days)	NH ₄ OH (once)	Sucrose (1 g.)	50.2	19.0	54.0	+++	+	++	++*
CO ₂ (3 days)	NH ₄ OH (twice)	Sucrose (1+1 g.)	72.8	21.4	46.3	+++	0	+	+++
Air	CaCO ₃ continuously	Sucrose (1+1 g.)	—	—	57.2	+++	0	0	+++

* Also homologous series of oligosaccharides; probably the isomaltodextrins (see Turvey & Whelan, 1956).

disaccharide had an R_F value of 0.37, cf. isomaltose 0.37, maltose 0.44, cellobiose 0.42 and glucose 0.65. A series of higher oligosaccharides was also present in the chromatograms from the partial hydrolysates and appeared to be identical with the series produced from the leuconostoc dextran (cf. Turvey & Whelan, 1957). Using the benzylamine technique the trisaccharide of the series had an R_F value of 0.20 (cf. isomaltotriose 0.20).

Total acid hydrolyses of the dextrans from *Streptococcus bovis* strains I and 1010 Y gave 97.0 and 96.0% conversion to glucose, respectively. Their optical rotations were: I dextran, $[\alpha]_D^{20} + 190^\circ$ (C. 0.30) and 1010 Y dextran, $[\alpha]_D^{20} + 187^\circ$ (C. 0.36). During 96 hr. of oxidation, at 29° , with sodium metaperiodate dextrans I and 1010 Y consumed 2.00 and 1.94 mole periodate and produced 0.97 and 0.96 mole of formic acid/mole anhydro-glucose, respectively.

Other oligosaccharides in the cultures

In cultures producing dextran, in addition to fructose, a second reducing disaccharide was detected with the silver nitrate spray (see Table 3). This component had a value of $R_{glucose}$ in solvent (a); 0.56 (cf. sucrose 0.70; isomaltose 0.44). It gave a positive ketose test with naphthoresorcinol and β -indolyl acetic acid sprays when they contained 2N-HCl, but not when the acid was trichloroacetic acid. A small portion of this sugar was isolated on paper chromatograms and hydrolysed with oxalic acid (1%, w/v) at 100° for 2 hr. Paper chromatograms of the hydrolysate showed the presence of glucose and fructose and much unhydrolysed disaccharide.

Normally no sign of glucose could be detected on chromatograms of the culture fluid. *Leuconostoc* spp. produce definite traces of glucose because of their secretion of small amounts of a levansucrase. The complete absence of glucose suggests that *Streptococcus bovis* does not secrete such a levansucrase. In a few 3-day neutralized cultures, however (Table 3), a strong glucose component together with components corresponding to the isomaltose series of oligosaccharides (cf. Turvey & Whelan, 1957) was present on the chromatograms.

DISCUSSION

The chromatographic identification of isomaltose as the sole disaccharide in the polysaccharide hydrolysates indicates that the polyglucosan is in fact dextran; this is confirmed by the positive optical rotation of the polysaccharide. The $[\alpha]_D$ values, together with the periodate oxidation figures, are in agreement with the results that would be expected for a polymer closely resembling unbranched leuconostoc dextran (Barker *et al.* 1955). There is a real need for CO_2 for maximum dextran production and presumably for dextran-sucrase secretion.

The appearance of an oligosaccharide concurrently with dextran production is not altogether unexpected as such sugars have been reported to occur in leuconostoc cultures (Stodola, Koepsell & Sharpe, 1952). In this latter case they are believed to arise through fructose acting as an alternative glucosyl acceptor. Whether this is the case in *Streptococcus bovis* cultures awaits identification

of the sugar. Detection of large amounts of glucose and the isomaltose series of oligosaccharides in occasional cultures was unexpected and an exception to the general complete absence of glucose. It may be suggested that in these cultures the organism had died and autolysis of the cells had released an invertase which had produced sufficient glucose to act as an alternative glucosyl acceptor. Glucose does act as such an acceptor to give oligosaccharides when added to *Leuconostoc* cultures (Bailey, Barker, Bourne & Stacey, 1955). As *S. bovis* is capable of fermenting sucrose without producing dextran presumably it contains an invertase.

Given the right conditions there can be little doubt that *Streptococcus bovis* is practically as good a dextran producer as *Leuconostoc mesenteroides*. Since *S. bovis* has a higher optimum growth temperature than *L. mesenteroides* the former produces dextran at a faster rate. It is tempting to relate the almost invariable CO_2 requirement of *S. bovis* for good dextran production to the fact that this organism is in the main homofermentative (see Smith & Sherman, 1942), whereas *L. mesenteroides* is heterofermentative; i.e. produces its own CO_2 by hexose fermentation. One of our *S. bovis* strains (I), which had an absolute CO_2 requirement for dextran production, required only 0.005M-HCO_3^- to produce a good yield of dextran. It may well be that *S. bovis* strains with no such absolute CO_2 requirement are ordinarily sufficiently heterofermentative to provide enough CO_2 for limited dextran formation. The data of Smith & Sherman (1942) show that *S. bovis* on the whole is not quite as good a lactic acid producer as *S. lactis*. In the above cases, however, so little gas is produced that they would be reported as homofermentative in ordinary bacteriological tests with Durham tubes. Since most *S. bovis* strains seem able to use oxygen to some extent, it is essential to exclude air as far as possible if a real CO_2 requirement for dextran production is to be demonstrated. It is worth noting that a closed culture flask half full of medium contains enough oxygen, in the air space, to yield 0.01M-HCO_3^- in the medium, provided that all of the oxygen is used for the complete oxidation of a carbohydrate to CO_2 .

If *Streptococcus bovis* dextransucrase has the same properties as *Leuconostoc* dextransucrase, i.e. a pH optimum of 4.0–4.5 and a temperature optimum of 25–29° with destruction at 37° (Bailey *et al.* 1957) then good dextran yields can only be obtained when the enzyme is continuously produced. This can only occur during continuous growth and all of the evidence (Table 3) indicates that pH control between 6 and 7 is necessary to ensure this. The properties of cell-free enzyme preparations are being investigated in order to clarify these points.

An important question is whether the effect of CO_2 on dextran yield is due to CO_2 gas in solution, the bicarbonate ion, or both. According to Umbreit, Burris & Stauffer (1945) HCO_3^- cannot exist in solution at pH values below 5, nor can CO_3^{2-} really exist below pH 8. Further, H_2CO_3 hardly exists at any pH value. Therefore in the pH range under study either or both pCO_2 and HCO_3^- may be implicated (cf. Loomis, 1957; Salisbury & Vandemark, 1957; Whitehead, Jones & Robertson, 1958; for instances of the control of biological

phenomena by $p\text{CO}_2$). We have not yet been able to settle this question. *Streptococcus bovis* (strain I) would not grow in a sucrose-containing nutrient medium containing HCO_3^- through which a slow stream of CO_2 -free and O_2 -free hydrogen was bubbled for 1 day. The same batch of medium did however give good growth and dextran production in the same time in an anaerobic jar filled with H_2 . The culture which failed to grow in a stream of H_2 later grew when air was admitted to the culture flask. Presumably the medium in the latter instance had an initial $p\text{CO}_2$ of about 0.02 % by volume (0.00001 M) as have all undisturbed aqueous liquids in air at 30–40° regardless of pH and HCO_3^- concentration (cf. Loomis, 1957). It will obviously need much careful work to disentangle the effects of trace amounts of CO_2 in initiating growth on the one hand and of the larger amounts which encourage dextran production on the other hand.

It must be granted that the role of CO_2 in dextran sucrose formation, if such a role exists, is not obvious at first sight. It may be a physical one since we have confirmed the observations of Dain *et al.* (1956) that Tween 80 can replace CO_2 in anaerobic conditions in encouraging dextran formation. A second possibility is that CO_2 , at the higher concentration, inhibits all modes of sucrose fermentation operating with *Streptococcus bovis* save one, the dextran-sucrose pathway, which may be stimulated. This latter possibility seems to be the best working hypothesis at the moment (cf. Salisbury & Vandemark, 1957). Although traces of CO_2 are apparently necessary to initiate growth, higher concentrations do not increase it (see Table 3). An explanation of why CO_2 stimulates the secretion of dextran sucrose by *S. bovis* might throw considerable light on the mechanism of the secretion of trans-glucosidases of bacterial cells.

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A Diazotizable Amine Produced by Yeast: its Chemical Nature and Factors Affecting its Accumulation

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SUMMARY: The effect of area:volume ratio and composition of medium on the accumulation of diazotizable amine ('amine') and hypoxanthine by yeast 47 growing in biotin-deficient medium are reported. The 'amine', isolated from culture filtrates by mercury precipitation and separation on ion exchange resins, gave the Pauly reaction for imidazoles: on acid hydrolysis it yielded ribose, glycine, ammonia and formic acid, but no phosphate. These properties are consistent with the view that the 'amine' is 5-amino-imidazole riboside or a closely related compound. When the growth medium contained 500 μ g. L-aspartic acid/ml., instead of 'amine' and hypoxanthine, substances accumulated which on hydrolysis yielded adenine as the only purine. 'Amine' accumulation by yeast 47 may be a symptom of a derangement of purine metabolism resulting from a biotin-conditioned block in aspartate synthesis.

Previous papers from this laboratory (Chamberlain, Cutts & Rainbow, 1952; Chamberlain & Rainbow, 1954) reported that a diazotizable amine ('amine') and hypoxanthine accumulated in culture filtrates of *Saccharomyces cerevisiae* (yeast 47) after growth in a biotin-deficient defined medium containing L-methionine; 'amine' accumulation was invariably associated with the formation of a pink pigment. It was also shown that inclusion of adenine or aspartate in the medium prevented the accumulation of 'amine', the formation of which was interpreted as being symptomatic of a derangement in purine metabolism conditioned by biotin deficiency. The present paper reports studies of some factors which affect the formation of 'amine' and hypoxanthine by yeast 47, a method of preparing purified 'amine' and a preliminary study of the chemical nature of 'amine'.

METHODS

Test organisms and preparation of inocula

Saccharomyces cerevisiae (yeast 47) was used for all experiments on the accumulation of 'amine', and cultures were maintained on malt agar slopes as described by Chamberlain & Rainbow (1954). Inocula were prepared by transferring organisms from a slope to 6 ml. of defined medium and incubating at 28° for 16-20 hr. The organisms were then centrifuged off, washed three times with 6 ml. portions of sterile, 0.85% saline and resuspended in a further 6 ml. of the saline for use in inoculation.

The strain of *Lactobacillus brevis* (L4) used for the detection of glycine and serine, and for the microbiological assay of glycine was that described by Moore & Rainbow (1955): it was maintained, and inocula were prepared, as described by them.

Media

Saccharomyces cerevisiae. Experiments with yeast 47 were carried out either in the defined medium I, having the composition given by Chamberlain & Rainbow (1954) or in medium II, differing from medium I in containing 10 times less $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (25 $\mu\text{g.}/\text{ml.}$). Both media contained 20 $\mu\text{g.}$ of D-biotin and 500 $\mu\text{g.}$ of DL-methionine/ml. However, for the preparation of inocula, methionine was omitted and the concentration of biotin was increased to 1 m $\mu\text{g.}/\text{ml.}$

Lactobacillus brevis L4. The medium described by Chamberlain & Rainbow (1954) was used with the following modifications: (a) 2% glucose was replaced by a mixture of 1% each of glucose and L-arabinose; (b) sodium acetate was omitted; (c) 10 $\mu\text{g.}$ each of adenine and guanine were added/ml. of medium; (d) casein acid hydrolysate was replaced by a mixture of amino acids as follows (mg./100 ml.): DL-alanine, 20; L-arginine hydrochloride, 31; L-asparagine, 10; L-aspartic acid, 7; L-cystine, 5; glycine, 10; L-glutamic acid hydrochloride, 18; L-histidine hydrochloride, 6; DL-isoleucine, 19; L-leucine, 16; L-lysine hydrochloride, 18; DL-methionine, 7; DL-phenylalanine, 16; L-proline, 49; DL-serine, 15; DL-threonine, 21; DL-tryptophan, 9; L-tyrosine, 9 and DL-valine, 31. For the detection of serine, DL-serine was omitted and for the detection and assay of glycine, glycine was omitted from this basal medium.

Growth of cultures

Tube cultures (6 ml.) were carried out as described by Northam & Norris (1951). Incubation was at 21° or 25° (yeast 47) or at 28° (L4). Each tube was inoculated with one drop (c. 0.03 ml.) of suspension. Growth was measured as dry weight of yeast 47/ml. derived from Spekker (Hilger, London) readings by reference to a previously determined calibration curve.

Larger cultures (40–700 ml.) of yeast 47 were grown in conical flasks or, in most cases, in 'Glaxo' culture vessels having a capacity of 2.5 l. These cultures were inoculated with a volume of inoculum proportionately larger than that used in tube tests (e.g. 3 ml. of suspension for a 500 ml. culture) except as indicated in the Results section.

Analytical methods

Diazotizable amine ('amine'). This was determined by the Bratton & Marshall procedure as described by Chamberlain & Rainbow (1954). Values quoted are the Spekker readings thus obtained.

Amino acids. These were detected qualitatively by paper chromatography (Chamberlain & Rainbow, 1954). Glycine and serine were distinguished with L4, which requires both amino acids for growth during the first 72 hr. Glycine in 'amine' hydrolysates was assayed by a similar technique in a medium to which serine was added; the approximate amount of glycine in the test sample was obtained by reference to a standard curve obtained with known amounts of glycine.

Imidazole derivatives. These compounds were detected by Koessler &

Hanke's (1919) modification of the Pauly reaction when in solution or by the modification introduced by Ames & Mitchell (1952) when on paper chromatograms.

Purine derivatives. After separation by paper chromatography purines were detected by ultraviolet photography. In culture filtrates, they were determined by ultraviolet spectrophotometry. These procedures were carried out as described by Chamberlain & Rainbow (1954).

Ion exchange. Conventional techniques were used, the resins having been taken through a depletion and regeneration cycle before use in the columns. In the process of purification of 'amine', the weakly acidic cation exchanger, Amberlite IRC-50, in the hydrogen form, and the weakly basic anion exchanger Amberlite IR-4B, in the free base form, were used. Further details are given in the Results section.

Quantitative analyses of 'amine'. Preparations of 'purified amine' were dried *in vacuo* to constant weight at a temperature below 40°. The dried 'amine' was then dissolved in 1.00 ml. of distilled water and a sample of 15 μ l. removed and diluted to 6.0 ml. for determination of diazotizable amine.

The ash was determined in a second portion (250 μ l.) of the solution of 'purified amine'; it was carefully evaporated to dryness on a piece of tared platinum foil and then heated to dull red heat until constant weight was attained.

Pentose was determined by Drury's (1948) orcinol method. Optical densities of the colour produced by the reaction were read at 670 m μ in the Unicam S.P. 500 quartz spectrophotometer (Unicam Instruments Ltd, Cambridge), the content of pentose in test solutions being calculated by reference to a calibration curve constructed by submitting known amounts of D-ribose to the same procedure. For determinations on the above solution of 'purified amine', samples of 10 μ l. were used.

The remaining 'amine' solution was hydrolysed by heating on a boiling water bath for 2 hr. with an equal volume of 4N-H₂SO₄. Determinations as follows were made on the hydrolysate.

Total nitrogen was determined on samples (0.10 or 0.20 ml.) of the hydrolysate by a conventional micro-Kjeldahl procedure.

For the determination of formic acid, 0.20 ml. of 'amine' hydrolysate was introduced into the micro-Kjeldahl apparatus, followed by 1 ml. of syrupy phosphoric acid (A.R.) and 0.8 ml. of distilled water. Steam was admitted and 20 ml. of distillate collected. To the distillate was added 0.25 ml. of 0.1N-Na₂CO₃ solution and 2.50 ml. of standard KMnO₄ solution (0.02N) and the mixture was heated for 15 min. at 80–90°. After acidification with 0.4 ml. of 10N-H₂SO₄, an excess (3.00 ml.) of 0.02N-sodium oxalate solution was added and the excess determined by titration against standard (0.02N) KMnO₄ solution. From the difference between this titre and that obtained when 0.20 ml. of distilled water was substituted for 'amine' hydrolysate in the same procedure, the amount of formic acid in the test sample was calculated. This procedure gave almost theoretical results when applied to solutions containing known amounts of formic acid.

Ammonia-nitrogen was determined on the residue remaining after distilling off the formic acid; the solution remaining in the micro-Kjeldahl apparatus was made alkaline by the addition of 4 ml. of 40 % NaOH solution and distillation was repeated. The ammonia distilled was trapped in 5 ml. of saturated boric acid solution (8 ml. of distillate being collected) and the ammonia present titrated against standard (0.01 N) HCl. Titres were corrected for the appropriate blank determinations.

Glycine-nitrogen was determined as the difference between total-nitrogen and ammonia-nitrogen.

RESULTS

Factors influencing the accumulation of 'amine' and hypoxanthine

Rate of formation of 'amine' and hypoxanthine. This was examined by growing yeast 47 in 40 ml. portions of medium I in 100 ml. conical flasks. One culture was removed daily for determination of 'amine' and hypoxanthine, so that the changes in area:volume ratio entailed by successive withdrawals of samples from a single culture was avoided. The results (Fig. 1) showed that 'amine' concentration reached a maximum after 5 days and diminished thereafter, whereas hypoxanthine continued to accumulate throughout the experiment (9 days), its concentration after 5 days being *c.* 18 $\mu\text{g./ml.}$

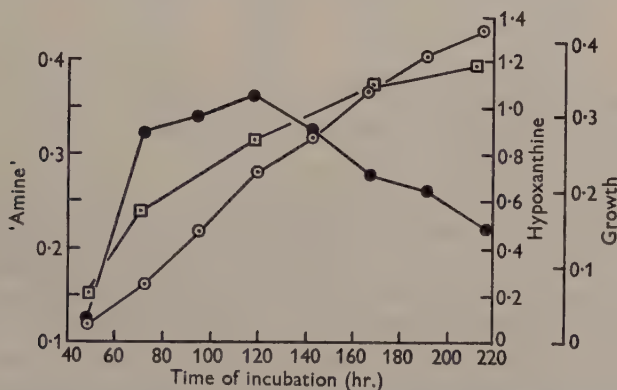


Fig. 1. Rate of formation of 'amine' and hypoxanthine in 40 ml. cultures of yeast 47 grown at 21°; ●, 'amine' as Spekker readings of the colour produced from unpurified culture filtrates in the diazotization reaction; ○, hypoxanthine as optical density at 250 $m\mu$; □, growth as mg. dry yeast weight/ml.

Effect of the ratio of surface to volume. For cultures grown in medium I in culture vessels, the amount of 'amine'/ml. of culture filtrate increased as the area/volume ratio was increased from 0.32 to 0.76, but hypoxanthine accumulation was unaffected. For the preparation of 'purified amine', 500 ml. in a culture flask was chosen as a convenient culture volume consistent with high yields of 'amine'.

Concentration of media constituents. The yield of 'amine' was decreased 19 % by decreasing the concentration of glucose to one-tenth; this paralleled the

decrease in growth. A similar decrease in concentration of *p*-AB reduced the yield of 'amine' and hypoxanthine by *c* 25% without affecting growth. A ten-fold diminution in the concentration of trace salts was accompanied by a decrease of 11% in the yield of 'amine', while diminution of the concentration of CaCl_2 increased the yield by 13% and that of hypoxanthine by 10% without affecting growth. Medium II contained Ca at this reduced level and was used to obtain material for the preparation of purified amine.

Table 1. *Effect of organic acids on 'amine' formation*

Tube tests at 25° for 4 days; organic acid supplements were adjusted to pH 5.0 with KOH solution and (except lactate) tested at a concentration of 0.05M; concentration of lactate as in medium I; growth [column (1)] as mg. yeast dry weight/ml.; column (2) shows 'amine' as Spekker readings of the colour of diazotized and coupled 'amine'; column (3) shows 'amine'/mg. yeast dry weight = column (2)/column (1).

Acid supplement	Medium I			Medium I without lactate		
	Growth (1)	'Amine'		Growth (1)	'Amine'	
		(2)	(3)		(2)	(3)
None	0.294	0.197	0.67	0.260	0.292	1.12
Acetic	0.260	0.054	0.21	0.242	0.098	0.41
Cisaconitic	0.268	0.100	0.37	0.268	0.077	0.30
Citric	0.277	0.206	0.74	0.260	0.311	1.19
Formic	0.074	0.030	0.41	0.088	0.020	0.23
Fumaric	0.409	0.197	0.48	0.388	0.297	0.76
Lactic	—	—	—	0.294	0.197	0.67
L-Malic	0.285	0.195	0.68	0.268	0.266	1.00
α -Oxoglutaric	0.242	0.0	0.0	0.234	0.0	0.0
Succinic	0.285	0.150	0.71	0.294	0.210	0.71

Effect of organic acids. These were studied in tube tests in medium I with and without lactate. The results (Table 1) show that, with the exception of citrate and L-malate, all the acids tested inhibited 'amine' formation per unit cell weight in both media. α -Oxoglutarate (0.05M) completely inhibited the formation of 'amine' but only slightly inhibited growth. Formate inhibited growth and 'amine' formation, whilst fumarate stimulated growth but had no effect on the level of 'amine'.

Organisms grown in the presence of 0.05M α -oxoglutarate lacked the pink colour invariably associated with those harvested from culture filtrates containing 'amine'. However, the concentration of α -oxoglutarate (0.05M) required completely to prevent the formation of 'amine' was much greater than that of adenine (0.0037M) or of L-asparagine (500 $\mu\text{g.}/\text{ml.}$ or 0.0038M, Fig. 2) required to produce the same result.

The effect of lactate, which is a normal constituent of the medium used for 'amine' formation, requires special mention. Omission of lactate led to the accumulation of appreciably greater concentrations of 'amine' and appeared to offer a means by which increased yields of 'amine' might be obtained. However, tests on the culture vessel scale showed that the amount of 'amine' precipitable by mercuric acetate from lactate-free media was only one-third

that obtained from media with lactate. Lactate was therefore retained as a constituent of the medium used for 'amine' preparation. Citrate was ineffective in replacing lactate in this respect.

Effect of aspartate on the accumulation of 'amine' and hypoxanthine

Effect on 'amine'. Chamberlain & Rainbow (1954) showed that the addition of 500 μ g. of L-aspartic acid/ml. to a culture of yeast 47 grown under conditions otherwise suitable for 'amine' formation almost completely prevented 'amine' accumulation. Table 2 shows that D-aspartic acid was also effective in this respect, but, unlike the L-isomer, it had no effect on the course of growth.

Table 2. *Effect of aspartate on growth of, and 'amine' formation by, yeast 47*

Tube tests in medium I at 25°; growth as mg. yeast dry weight/ml.; 'amine' as Spekker readings of the colour produced from unpurified culture filtrates in the diazotization reaction; aspartic acid supplements (adjusted to pH 5.0 with KOH solution), 500 μ g. of the D-, L- or DL- forms/ml.

Aspartic acid supplement	Growth				'Amine' 96 hr.
	Time (hr.)				
	24	48	72	96	
Nil	0.023	0.108	0.187	0.268	0.212
L-	0.136	0.187	0.187	0.187	0.059
D-	0.028	0.122	0.187	0.268	0.069
DL-	0.129	0.202	0.194	0.194	0.058

The minimum concentration of L-aspartic acid necessary to effect maximum inhibition of 'amine' formation was *c.* 500 μ g./ml. (Fig. 2). The same experiment showed that much smaller concentrations (*c.* 30 μ g./ml.) of L-aspartic acid stimulated 'amine' accumulation.

Effect on hypoxanthine. Cultures (500 ml.) of yeast 47 in medium I and in medium I supplemented with 500 μ g. of L-aspartic acid/ml. were grown at 21° for 5 days. After removing the cells, a concentrate containing all the 'amine' and hypoxanthine was prepared from each culture by precipitation with mercuric acetate at pH 4.4, decomposition of the mercury complex with H₂S and concentration of the resultant solution, as in the usual procedure for preparing 'amine'. Ultraviolet spectroscopy showed that, in the presence of L-aspartic acid, a material with $E_{\text{max.}}$ 256 m μ accumulated, whereas in the absence of L-aspartate a material, presumably hypoxanthine (Chamberlain & Rainbow, 1954) with $E_{\text{max.}}$ 250 m μ was obtained. Paper chromatography revealed that no spot corresponding to hypoxanthine was given by the concentrate derived from the aspartate-supplemented culture. Instead, ultraviolet-absorbing substances, which were absent from the concentrate derived from the unsupplemented culture, were present. One of these substances had a chromatographic mobility considerable greater than that of hypoxanthine, and at least three others had mobilities much less than that of hypoxanthine. These new ultraviolet-absorbing substances were also chromatographically distinct from adenine and guanine.

A portion (1 ml.) of a concentrate obtained from an aspartate-supplemented culture was hydrolysed with 1N-H₂SO₄ for 2 hr. at 100°; after neutralizing with Ba(OH)₂ and centrifuging, the supernatant was evaporated *in vacuo* to 1 ml. This solution contained free reducing sugar, and paper chromatography showed that the ultraviolet-absorbing substances of the unhydrolysed concentrate had been replaced by a single spot, chromatographically identical with adenine. This spot, and one of authentic adenine,

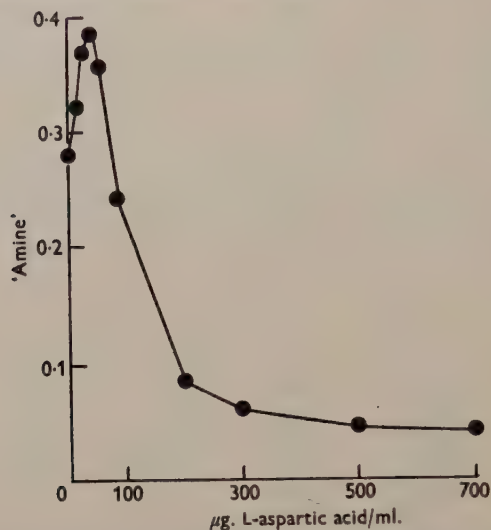


Fig. 2. Effect of concentration of L-aspartic acid on 'amine' formation. Tube tests in medium II at 21° for 5 days; 'amine' as Spekker readings of the colour produced from unpurified culture filtrates in the diazotization reaction.

were eluted from the paper and examined spectrophotometrically; both materials exhibited peak absorption at 262 m μ and their absorption ratios, measured at 5 m μ intervals between 235 and 290 m μ , were the same within 2%. The ultraviolet absorbing substances in the hydrolysate therefore appeared to be adenine, from which it may be inferred that the new ultraviolet-absorbing substances which accumulated in cultures containing L-aspartate were derivatives of adenine.

Effects of other substances. The inhibitory effects of norleucine, norvaline, adenine and aspartic acid on 'amine' formation were described by Chamberlain & Rainbow (1954). Tube tests in medium I at 21° for 5 days showed that the addition of single pyrimidines (25 μ g./ml. of uracil, thymine, cytosine or orotic acid) enhanced 'amine' formation by *c.* 5–10%, but growth was unaffected. When all the pyrimidines were present together, 'amine' formation was enhanced by *c.* 20%.

Preparation of 'purified amine'

The early stages of the preparation were based on the procedure of Chamberlain & Rainbow (1954) with the following modifications: (a) medium II was used, each culture vessel containing 500 ml., and (b) each culture was inocu-

lated with 1 ml. of washed yeast suspension. 'Amine' was precipitated with mercuric acetate and the precipitate washed as previously described. The washed precipitate from each culture was then shaken into suspension in 32.5 ml. of 0.001N- H_2SO_4 and decomposed by passing water-washed H_2S through the suspension for 45 min. After removing excess H_2S in a vigorous current of air, the pH was adjusted to a value of *c.* 9.0 with 3.5 ml. of saturated $\text{Ba}(\text{OH})_2$. The precipitate (containing HgS , BaSO_4 and $\text{Ba}_3(\text{PO}_4)_2$) was removed by centrifuging, washed once with distilled water and the combined supernatants adjusted to pH 4.0 with 4.5 ml. of 0.1N- H_2SO_4 . The 'amine' solution, obtained after centrifuging off and washing the BaSO_4 thus precipitated, contained (as judged by diazotizability) 85% of the 'amine' present in the original culture filtrate and was stable on storage for 6 weeks at pH 4.0 at 4°. This improved recovery of 'amine' [Chamberlain & Rainbow (1954) obtained only a 49% recovery] appeared to be chiefly the result of the treatment at pH 9.0, which effected desorption of 'amine' from the HgS precipitated at pH 4.0. At this stage, the 'amine' solution contained also hypoxanthine, amino acids (chiefly methionine) and the anions sulphate and acetate, but not phosphate.

The 'amine' was separated from these contaminants on ion exchange resins. The 'amine'-containing solution (pH 4.0), derived from one 500 ml. culture, was percolated, at a flow rate of 100 ml./hr. through a column (40 × 1.6 cm.) containing 50 g. of *Amberlite* IRC-50 resin in the hydrogen form. This was followed by distilled water at the same rate until 250 ml. of total effluent had collected. Distilled water (1 l.) was then passed through the column at the rate of 200 ml./hr. After ensuring that the optical density at 250 m μ . of the last runnings of this effluent (which contained the hypoxanthine) was at a constant low level, the outlet jet of the column was immediately connected to the top of a column (9 × 1.1 cm.) containing 3 g. of *Amberlite* IR-4B resin in the hydroxyl form and 400 ml. of 0.005 N-HCl was allowed to flow through both resin beds in series at a flow rate of 200 ml./hr. Very dilute acid was used for this elution since it was known that 'amine' was particularly labile under acid conditions, treatment with 0.01 N-HCl for 2 hr. causing a 23% loss of diazotizability. The acid-free effluent was concentrated *in vacuo* at less than 35° to yield a yellowish, crystalline residue (6.5–15 mg. from each 500 ml. of original culture). This material, subsequently referred to as 'purified amine', was shown by paper chromatography to be substantially free from amino acids, and by ultraviolet spectroscopy to be free from hypoxanthine. 'Purified amine' was stored *in vacuo* in the dark; it became darker in colour on storage, especially if air was admitted.

Overall recoveries of 'amine' as 'purified amine' varied from about 30% to 80% as judged by diazotizability. Losses may have been caused by decomposition of 'amine' on the resin columns. Attempts to purify 'amine' further have been hampered by its lability, especially in the presence of air, in aqueous solutions having pH values outside the range pH 4–6. It has not been possible to recover 'amine' from sorption on resins other than weakly acidic cation exchangers and relatively little 'amine' survived chromatographic separation on columns of cellulose powder developed with *n*-butanol + acetic acid + water.

Analysis of 'purified amine'

Pentose content. Chamberlain & Rainbow (1954) have already suggested that 'amine' is implicated in purine biosynthesis and, since it is widely accepted that the latter proceeds at the nucleoside or nucleotide level, 'amine' was tested for pentose as follows.

(i) 'Purified amine' gave a positive reaction for pentose in the orcinol test.
(ii) No colour was formed when 400 μg . of 'purified amine' dissolved in 2 ml. of water was heated at 100° for 10 min. in Dische's (1955) test for 2-deoxy-pentoses. D-Ribose (50 μg) also failed to give the reaction, but 50 μg . of authentic D-2-deoxyribose gave a strong blue colour under the same conditions.

(iii) Pentose was present in 'purified amine' in a combined, non-reducing form, since 50 μg . of 'purified amine' as a spot on filter paper gave no pink colour when treated with aniline hydrogen phthalate at 100°.

(iv) 5 mg. of 'purified amine' was hydrolysed with 2 ml. of 1N-H₂SO₄ at 100° for 2 hr. The hydrolysate was neutralized with Ba(OH)₂ to pH 6.5, centrifuged and the supernatant evaporated *in vacuo* at 35° to c. 0.25 ml. This hydrolysate was not diazotizable by the Bratton & Marshall procedure, but it contained reducing sugar. The hydrolysate (20 ml.) was then chromatographed on paper with reference spots containing 10 μg . each of D-ribose, D-xylose and L-arabinose according to the procedure of Giovannozzi-Sermanni (1956). The spots, revealed by aniline phthalate, showed that the chromatographic mobility of the reducing sugar present in the hydrolysate corresponded closely with that of D-ribose.

Phosphate content. No phosphate was detected in 'purified amine' after digestion with HClO₄ according to the procedure of Allen (1940).

Pauly test. If, as suggested by Chamberlain & Rainbow (1954), 'amine' possesses an incomplete purine structure, it may be an imidazole derivative similar in structure to the recognized purine precursor, 4(5)-amino-5(4)-imidazolecarboxamide (AICA), from which, however, 'amine' is distinct (Chamberlain & Rainbow, 1954). The possible presence of the imidazole nucleus in 'amine' was therefore examined by application of the Pauly reaction. In this reaction, 50 μg . of 'purified amine' gave a purple colour, which faded within 5 min. to yellow similar to the blank. Under similar conditions, 40 μg . of histidine gave a permanent red colour and 40 μg . of AICA gave a permanent blue colour. Hypoxanthine (100 μg .) gave no reaction. The colour given by 'amine', although transitory, confirms the presence of the imidazole nucleus: a transitory colour is given by other imidazoles such as 4-ureido-5-imidazolecarboxylic acid (Rabinowitz & Pricer, 1956) and 5-amino-imidazole ribotide (Levenberg & Buchanan, 1957). That the substance giving the transient Pauly reaction was identical with the diazotizable substance was indicated by paper chromatography in which two spots of 'purified amine' (100 μg . each) were run side by side: after development in *n*-butanol + acetic acid + water (Chamberlain & Rainbow, 1954), the paper was cut longitudinally and one half sprayed with Bratton & Marshall reagents and the other with Pauly reagents. This procedure showed that the diazotizable sub-

stance and that giving a transient colour in the Pauly reaction were chromatographically identical.

Products of acid hydrolysis. 5 mg. of 'purified amine' was hydrolysed under reflux with 2 ml. of 1N-H₂SO₄ for 2 hr. at 100°. After adjustment to pH 6.5 with saturated Ba(OH)₂ solution and centrifuging, the clear supernatant was evaporated *in vacuo* to dryness at 40° and the residue was taken up in 0.25 ml. of distilled water. Portions of this solution were examined as follows.

(i) Paper chromatography (20 μ l. spots) in *n*-butanol + acetic acid + water revealed only one ninhydrin-reacting spot corresponding either to glycine or to serine. Further examination in a phenol + water solvent distinguished this material as glycine and not serine. Its identity as glycine was confirmed since the acid hydrolysate of 'purified amine' substituted for glycine, but not for serine, in the growth of *Lactobacillus brevis* L4 which requires both amino acids. By microbiological assay with L4, the approximate amount of glycine obtained after hydrolysing 2.4 mg. of a preparation of 'purified amine' was determined as 252 μ g. (Table 3).

Table 3. *Analysis of 'purified amine'*

Except as indicated, analytical methods were as described in the 'Methods' section. Figures in the column headed ' μ mole' are calculated on an ash-free basis.

	Preparation no.							
	I		II		III		IV	
	μ g.	μ mole	μ g.	μ mole	μ g.	μ mole	μ g.	μ mole
'Purified amine'	2400	—	13220	—	19910	—	29510	—
Ash	1005	—	2751	—	5595	—	4604	—
Ribose	314	2.1	5360	36	2850	19	2387	16
Formic acid	—	—	—	—	1991	44	2401	52
Total-N	—	—	1872	134	2528	181	5774	412
Ammonia-N	142*	10.1	1443	103	1764	126	4900	350
Glycine-N	47†	3.4	429	31	764	55	874	62

* By Nesslerization.

† By microbiological assay.

(ii) The presence of ammonia in the hydrolysate was shown with Nessler's reagent. By comparing the colour produced in this reaction with that produced from known ammonium chloride standards, the approximate amount of ammonia-N yielded by acid hydrolysis of 'purified amine' was determined (Table 3).

(iii) Three drops of the hydrolysate were acidified and then reduced by adding magnesium powder. The reduced product gave a positive chromotropic acid test for formaldehyde.

The results described in (i) and (ii) above showed that acid hydrolysis of 'purified amine' yielded glycine and ammonia. There was also evidence [(iii) above] that formic acid was also produced. The formation of these products, known to arise by hydrolytic degradation from certain imidazole derivatives (Hunter, 1936), is confirmation that the imidazole nucleus may form part of the structure of 'amine'.

Chromatographic behaviour. When relatively large amounts (50–60 μ g.) of

'purified amine' were chromatographed on paper, a second material, showing as a less intense red spot in the diazotization reaction, was apparent just below the main constituent. By development in *n*-butanol+acetic acid+water (4:1:5), the smaller spot became well separated from the larger one. That glycosidically bound pentose was associated only with the larger spot was shown by spraying replicate chromatograms with (i) 1% lead tetraacetate in benzene and (ii) aniline hydrogen phthalate. The material having a chromatographic mobility identical with the larger diazotizable spot gave a positive reaction in test (i), but test (ii) was negative, showing that neither material contained free reducing sugar.

Ultraviolet absorption. Figure 3 shows the ultraviolet absorption curve of 'purified amine'. The curve has a feeble peak at 238 $m\mu$.

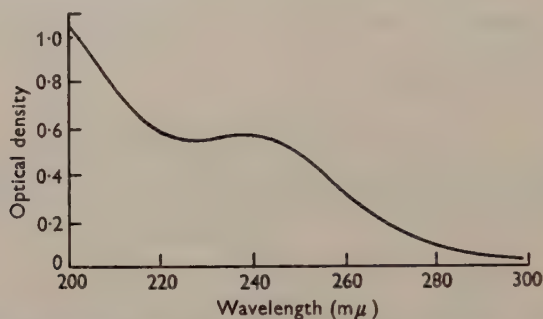


Fig. 3. Ultraviolet absorption curve of 'purified amine' (105 $\mu\text{g./ml.}$) in aqueous solution.

Quantitative analyses of 'purified amine'

Table 3 gives some representative results of analyses of preparations of 'purified amine'. Because of the instability of 'amine', analyses were performed on freshly prepared samples or on samples which had been stored *in vacuo* not longer than 24 hr.

The analyses show that the composition of 'purified amine' varies from preparation to preparation. Nevertheless, on acid hydrolysis, all preparations yielded substantial amounts of ribose, formate, ammonia and another nitrogen compound, presumed to be glycine from the evidence of paper chromatography and microbiological assay. Formate, ammonia and glycine are the products of acid hydrolysis of 5(4)-amino-imidazole (Hunter & Nelson, 1941). The presence of ribose in the hydrolysis products suggests that 'amine' may occur as the riboside of 5(4)-amino-imidazole, but the expected molar ratios of ribose:formate:glycine:ammonia:(1:1:1:2) are not realized in our analytical figures. On an ash-free basis, the molar proportions of glycine:formate in samples so far analysed for both of these substances (e.g. preparations nos. III and IV in Table 3) have proven to be approximately 1:1. However, the values for ammonia-N are too high and those for ribose are usually too low. Low values for ribose are possibly attributable to the presence in 'purified amine' of 'amine', not in the riboside form. This possibility is supported by the evidence of the chromatographic behaviour of 'purified amine'.

Our results suggest that our preparations usually contain both 'amine' and 'amine' riboside, contaminated with mineral and ammonium salts, the latter arising possibly from decomposition of 'amine' on the columns.

DISCUSSION

A number of known imidazoles yield glycine, ammonia and formic acid on acid hydrolysis (Hunter & Nelson, 1941; Hofmann, 1953). The formation of these products depends upon positions 1, 2 and 4 (or 5) being either unoccupied or occupied by labile substituents. Thus, 4(5)-amino-5(4)-imidazole-carboxylic acid yields CO₂ in addition to these products (Rabinowitz, 1956) but 4(5)-amino-5(4)-methylimidazole yields α -alanine, ammonia and formic acid (Fargher, 1920).

Nc1nc[nH]c1[C@@H]2O[C@H](CO)[C@@H](O)[C@H]2O

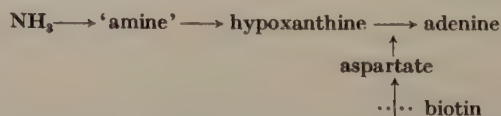
5-amino-imidazole riboside

The analytical figures (Table 3) show that the amounts of ribose, ammonia, formate and glycine are inadequate to account for all the material present in ash-free 'purified amine'. The discrepancy may be accounted for either by the presence of volatile salts in the preparation, or by the presence of an undetected labile substituent in position-4 (or 5). Such a substituent might be a carboxyl group, although this seems unlikely because of the behaviour of 'amine' on ion exchange resins.

The paper chromatographic behaviour of 'purified amine' suggests that the preparations contain diazotizable material both in the free and riboside forms. Both forms may be present in the original culture filtrates or free diazotizable amine may arise by rupture of the glycosidic bond during isolation. The diazotizable aglycone presumably would be retained by the resin and would appear in the final product together with the riboside.

The present work confirms the view of Chamberlain & Rainbow (1954) that the accumulation of 'amine' is a symptom of a disordered purine metabolism in yeast 47. Since the biosynthesis and transformations of purines are recognized to occur at the nucleoside or nucleotide level, it is not surprising that 'amine' is a riboside. The metabolic defect in question appears to be mainly an inability to aminate hypoxanthine to adenine.

The accumulation of 'amine' in cultures of yeast 47 is prevented by the addition of adenine or of aspartate (Chamberlain & Rainbow, 1954). The present work shows that, when 'amine' formation is depressed by addition of aspartate, adenine derivatives accumulate instead of hypoxanthine. These facts suggest that the accumulation of 'amine' and hypoxanthine by yeast 47 is the result of a biotin-conditioned deficiency of aspartic acid. In this connexion, the involvement of biotin in aspartate metabolism has been established for yeast (Winzler, Burk & du Vigneaud, 1944) and for lactic acid bacteria (Broquist & Snell, 1951), whilst Lichstein & Umbreit (1947) have established a role for biotin in amination reactions leading to aspartate formation. In yeast 47, aspartate may be the donor of the amino group attached to position-6 of the purine skeleton by means of which hypoxanthine is converted to adenine in reactions occurring presumably at the riboside or ribotide level. The following biosynthetic sequence may represent the course of events in yeast 47:



Other recent work supports the contention that aspartic acid is involved in the conversion of derivatives of hypoxanthine to derivatives of adenine: thus Abrams & Bentley (1955) showed that aspartate was required for the amination of inosinic acid to adenylic acid by rabbit bone marrow. Again, Lieberman (1956) showed that in the transformation of inosine 5'-phosphate to adenosine 5'-phosphate by extracts of *Escherichia coli*, adenylosuccinic acid (6-aspartylpurine nucleotide), already isolated by Carter & Cohen (1955) with the aid of an enzymic extract obtained from baker's yeast, was intermediate.

In addition to its role in the biochemical transformation of purine derivatives, aspartate also contributes the nitrogen in position-1 of the purine skeleton (Levenberg, Hartman & Buchanan, 1956).

The present results fit in well with current ideas of purine biosynthesis by Levenberg & Buchanan (1957). The properties of 'amine' resemble closely those of 5-amino-imidazole investigated by those workers and those of the diazotizable amines, considered to have a similar structure, examined by Love & Gots (1955) and by Moat, Wilkins & Friedman (1956).

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B₁₂-Vitamins and Growth of the Flagellate *Ochromonas malhamensis*

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SUMMARY: The flagellate *Ochromonas malhamensis* requires vitamin B₁₂ and responds only to those natural forms of the vitamin which are active also for higher animals. The relation between the rate of growth and the concentration of cyanocobalamin is described by an equation of the form of an adsorption isotherm. The 'inactive' analogues, pseudovitamin B₁₂ and Factor A, were taken up by *O. malhamensis* to about the same degree as cyanocobalamin, and inhibited competitively the growth response to cyanocobalamin, apparently by blocking a cell mechanism for 'binding' the vitamin.

At least half the marine algal flagellates and diatoms which have so far been studied in pure culture require an exogenous supply of vitamin B₁₂ (Droop, 1957 *a*), and in the natural orders Chrysomonadina and Euglenida the requirement is probably characteristic (cf. Hutner, in Ford & Hutner, 1955). That so many of these planktonic organisms require vitamin B₁₂ explains the present keen interest among marine biologists in the ecological role of the vitamin, particularly about the question whether the concentration of available vitamin B₁₂ in the seas can fall so low as to limit the development of plankton communities. Droop (1957 *b*) calculated that, on present evidence, there must be at all times more than enough vitamin B₁₂ in the sea for the crops which are encountered. Daisley (1957) argued that this view is based on a questionable assumption, namely, that determinations of the potency of vitamin B₁₂ for a few species, made under the conditions of laboratory pure culture, can be applied in an assessment of the natural situation. Daisley urged the need for much more information to be collected before any conclusion is drawn about the status of vitamin B₁₂ in marine ecology. The present paper illustrates that the factors involved are indeed complex, and is concerned with the influence of vitamin B₁₂ and of certain of its natural analogues on the growth of the freshwater chrysomonad *Ochromonas malhamensis*.

Ochromonas malhamensis is facultatively photosynthetic, and when grown in the light has relatively simple nutritional needs (Hutner, Provasoli & Filfus, 1953). For optimal growth in darkness its requirements are more complex, and have been worked out in some detail (Ford, 1953). The organism has an absolute and specific need for vitamin B₁₂, and responds selectively to the natural forms of the vitamin which are active also for higher animals (Coates & Ford, 1955).

METHODS

Test media. Most of the experiments involved growing *Ochromonas malhamensis* in darkness in a medium of the composition shown in Table 1. This medium allowed rapid and abundant growth, and was complete in the sense that the response to limiting amounts of cyanocobalamin was not enhanced by the presence of a variety of crude natural extracts. For incubations in light the medium was modified by the omission of casein hydrolysate, tryptophane, methionine and cystine, and by the inclusion of 0.3 g. NH_4Cl /100 ml. medium.

Table 1. *Composition of culture medium*

	(g.)		(mg.)
Acid hydrolysed casein, 'vitamin free'*	5	DL-Methionine	200
Glucose	10	L-Cystine	100
Diammonium hydrogen citrate	0.8	m-Inositol	10
KH_2PO_4	0.3	Choline chloride	2
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2	Thiamine	2
CaCl_2	0.15	p-Aminobenzoic acid	1
'Metals' solution†	10 ml.	NaCN	2
	(mg.)		
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	50	Biotin	10 μg .
DL-Tryptophane	100	Tween 80	1 ml.

The pH value of the solution was adjusted to 5.5, the volume being made up with distilled water finally to 1000 ml.

* Allen and Hanburys Ltd.

† After Hutner (private communication). The solution had the following composition: ethylenediamine tetra-acetic acid, 5 g.; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 6.15 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 11 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g.; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.04 g.; H_3BO_3 , 0.06 g.; KI, 0.001 g.; water to 1000 ml.

Maintenance of organism and preparation of inocula. The stock cultures were maintained in the basal medium (Table 1), supplemented with 0.1 μg . cyanocobalamin/ml. and autoclaved at 115° for 15 min. in 10 ml. portions held in 50 ml. conical flasks. They were incubated at about 27° under constant illumination at 240 foot-candles, and subcultured from 0.5 ml. inoculum at 4-day intervals. Grown under these conditions the cultures reached about 5,000,000 organisms/ml. at 4 days, and were used at this stage as inocula for the growth tests to be described. Some of the experiments required relatively large amounts of culture. These were grown as described above, but were grown in larger flasks ('penicillin bottles') each containing 400 ml. of medium, and from proportionately larger inocula.

Growth-rate tests. Most of the growth-rate tests were incubated in the dark. They were set up in optically matched Pyrex test tubes (19 × 150 mm.) each containing 5 ml. test medium. The tubes were plugged with cotton wool and autoclaved for 10 min. at 115°. After cooling to room temperature each tube was inoculated with one drop of undiluted 4-day culture and placed in a shaking machine in an air incubator operating at 29°. After the chosen time intervals the cultures were removed from the incubator and their relative optical densities (at 580 $\mu\mu$) determined in a Lumetron model 400 A photometer.

All the cultures were examined microscopically, and in some the mean diameters of the organisms were determined and counts made.

Tests of growth in the light were done in the modified medium (see above), and were set up in 50 ml. flasks and incubated at 27° in a thermostatically-controlled cabinet, uniformly illuminated at 180 foot-candles. Apart from that, they were performed as described above for the tests incubated in darkness.

Sources of B₁₂-vitamins. Cyanocobalamin was kindly provided by Glaxo Laboratories (Greenford) Ltd; vitamin B_{12 III} was kindly given by Dr K. Bernhauer (Biochemisches Laboratorium der Aschaffenburger Zellstoffwerke, A.G., Stockstadt-am-Main), and pseudovitamin B₁₂, Factor A, Factor B, Factor C and Factor D by Drs E. S. Holdsworth and J. W. G. Porter. All these analogues of vitamin B₁₂ occur naturally. They are red, cobalt-containing substances and are closely related chemically to cyanocobalamin (for review see Kon, 1955). The differences in chemical structure, where these are known, are confined to the nucleotide portion of the molecule. Thus, cyanocobalamin nucleotide is 5:6-dimethylbenziminazole- α -D-ribofuranose phosphate, and vitamin B_{12 III} nucleotide is the 5-hydroxybenziminazole ribotide. In pseudovitamin B₁₂ the nucleotide is based on adenine, and in Factor A on 2-methyladenine. Factor B has no nucleotide, and constitutes the non-nucleotide portion of the molecule in the above three analogues. The structures of Factors C and D are not yet known.

RESULTS

Vitamin B₁₂ and the growth of Ochromonas malhamensis

Fig. 1 shows the progression of growth in the dark (plotted as log₁₀ optical density readings) with time of incubation at different concentrations of cyanocobalamin. Growth with no added cyanocobalamin was very slight, and can be attributed to carry-over in the inoculum, and in part also to some vitamin contained in the 'vitamin-free' casein hydrolysate used in the medium.

In actively growing cultures the organisms were highly motile and ranged in diameter from *c.* 5 to 15 μ , averaging about 10 μ . Older cultures tended increasingly to contain cell debris and large agglomerates of non-motile small organisms, and accurate counting was not possible. In young cultures the counts were roughly proportional to the optical density readings, but the latter were preferred as a measure of total growth product. Measurements of the optical densities of serial dilutions of cultures of *Ochromonas malhamensis* at different stages of growth showed that they obeyed Beer's law within the limits of accuracy of the photometer.

No abnormalities in morphology were noticed as being characteristic of vitamin B₁₂ depletion. Even after incubation for 138 hr. the organisms grown with no added vitamin appeared quite normal, and were all highly motile. These cultures were remarkable only for their very slow rate of growth. In contrast, cultures grown at high concentrations of cyanocobalamin grew very rapidly to the stage of 'ageing'. At this stage, numbers increased faster than the rate of production of new cell material (as measured by optical density),

and the organisms diminished in size from a volume of *c.* $650\mu^3$ to *c.* $200\mu^3$. These aged organisms were non-motile and characteristically acorn-like in appearance; their contents were granular and confined at one end. On further incubation the organisms autolysed completely, and the cultures became quite clear. But this process of ageing in the cultures is only indirectly relevant to the metabolism of vitamin B₁₂, and can probably be attributed to the exhaustion of other nutrients in the culture medium.

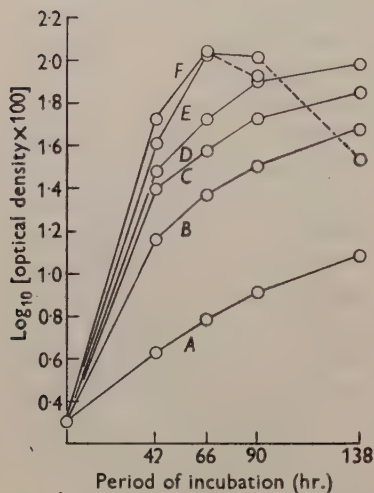


Fig. 1

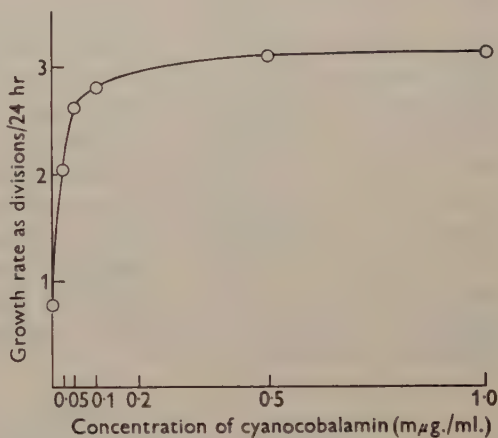


Fig. 2

Fig. 1. Influence of the cyanocobalamin concentration on the growth of cultures of *Ochromonas malhamensis*. A, no added cyanocobalamin; B, 0.025 $\mu\text{g./ml.}$; C, 0.05 $\mu\text{g./ml.}$; D, 0.1 $\mu\text{g./ml.}$; E, 0.5 $\mu\text{g./ml.}$; F, 1.0 $\mu\text{g./ml.}$

Fig. 2. Influence of cyanocobalamin concentration on the rate of growth of *Ochromonas malhamensis* during the first 42 hr..

It seemed probable, from Fig. 1, that growth of the cultures was exponential only during the early hours of incubation. Plotting the relative growth rates shown during the first 42 hr. against the concentrations of cyanocobalamin gives a conventional rate/concentration curve (Fig. 2) which, according to Hinshelwood (1946), follows an equation of the form of an adsorption isotherm. It is described by the equation $K/K_{\text{max.}} = C/C_1 + C$, where K is the relative growth rate and $K_{\text{max.}}$ the rate when $C = \infty$; C is the concentration of cyanocobalamin, and C_1 is that value of C at which the value of K lies half-way between zero and $K_{\text{max.}}$. $1/K$ plotted against $1/C$ gives a straight line in this equation (Fig. 3). From this $K_{\text{max.}}$ can be read off as 3 divisions/24 hr., and C_1 (the concentration of cyanocobalamin which allows half maximal growth-rate) as 0.000013 $\mu\text{g./ml.}$ Under the conditions used, the rate/concentration relation appeared to be logarithmic. However, Hinshelwood (1946) showed that at very low concentrations, the growth rate (K) would become proportional to the concentration of cyanocobalamin (C).

Droop (1957*b*) pointed out that the requirements of several flagellates for vitamin B₁₂ are very low, and are quantitatively similar. *Monochrysis lutheri*, *Euglena gracilis* and a *Stichococcus* sp. each required about 3 molecules of cyanocobalamin/ μ^3 of living alga. From the present experiments the minimal requirement for *Ochromonas malhamensis* is found to be of the same order. When grown in the light, the organisms required about 1 molecule of cyanocobalamin/ μ^3 ; grown in darkness they required about 3 molecules/ μ^3 . But

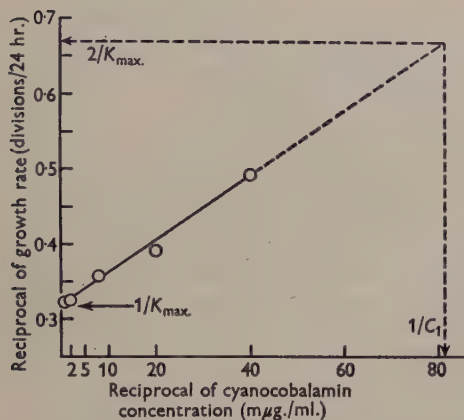


Fig. 3

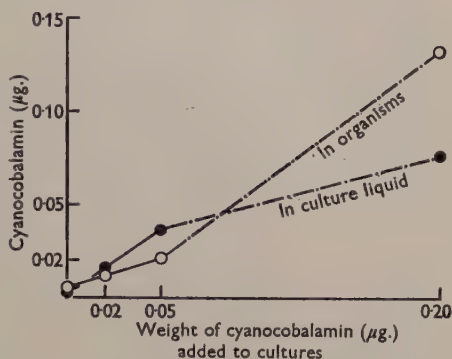


Fig. 4

Fig. 3. Influence of cyanocobalamin concentration on the rate of growth of *Ochromonas malhamensis*: $1/K$ against $1/C$.

Fig. 4. Uptake of cyanocobalamin added in graded amounts to portions of a culture of *Ochromonas malhamensis*.

whether the similarity of all these estimates is more than fortuitous is open to question. In *O. malhamensis* at least, the requirement varies with the conditions of culture. For example, Hutner, Sanders, McLaughlin & Scher (1957) showed that over the temperature range 30–37° the requirement for cyanocobalamin increased 500-fold; and even 30° is much higher than the temperatures prevailing in Malham Tarn, whence *O. malhamensis* was first isolated. However, taking Droop's assumption that requirements as low as 3 molecules of cyanocobalamin/ μ^3 are fairly typical (and in the natural environment one can conceive the possibility of even lower requirements), his estimate that shortage of vitamin B₁₂ does not limit the size of marine plankton crops seems to ignore what is clearly an important consideration, namely, that of the influence of the concentration of vitamin B₁₂ upon the rate of growth.

The uptake of B₁₂-vitamins by Ochromonas malhamensis

Uptake of cyanocobalamin. Cyanocobalamin was added in graded amounts to 40 ml. portions of a culture of *Ochromonas malhamensis*, grown with limiting amounts of cyanocobalamin, as described on page 162. The portions were shaken for 2 hr. at 30° and centrifuged; organisms and supernatant fluids were then extracted and assayed separately for cyanocobalamin, with *Lacto*-

bacillus leichmannii as assay organism (cf. Coates *et al.* 1953). The distribution of the added vitamin between organisms and culture medium is shown in Fig. 4.

From this and similar experiments it was evident that, at 'physiological' concentrations, the uptake of cyanocobalamin was proportional to the concentrations added. At higher concentrations the uptake was somewhat more

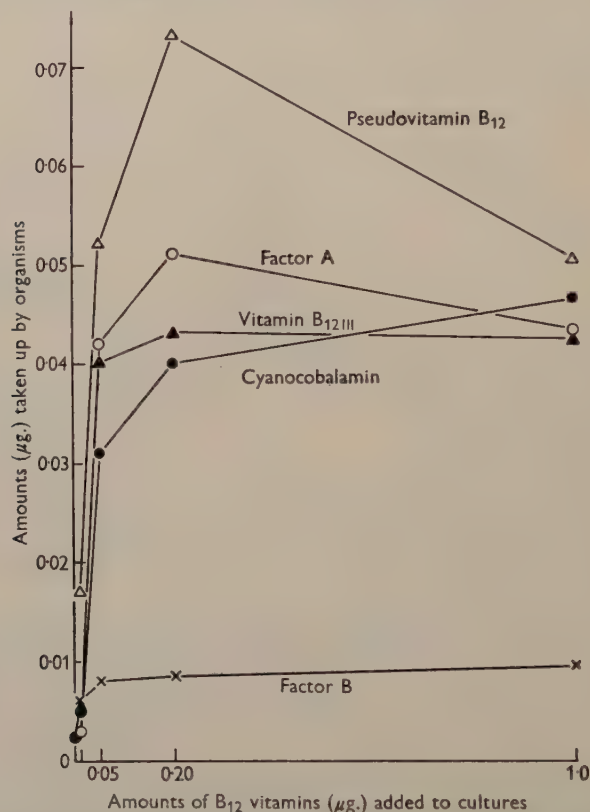


Fig. 5. Uptake of different B₁₂-vitamins, added in graded amounts to equal portions of a culture of *Ochromonas malhamensis*.

efficient, and was again proportional to the amounts added. A transition from 'inefficient' to 'efficient' uptake is reflected in an abrupt increase in the slope of the uptake curve, and is probably associated with the property of the culture fluid of 'binding' vitamin B₁₂ (see p. 167).

Uptake of vitamin B₁₂ analogues. Of the naturally occurring analogues of vitamin B₁₂, *Ochromonas malhamensis* responds only to vitamin B₁₂III. The other natural analogues have little or no activity, and are represented in this work by Factor A, Factor B and pseudovitamin B₁₂ (for reviews of literature on B₁₂ vitamins, see Kon, 1955; Ford & Hutner, 1955). To determine whether these biological properties are related to the efficiencies with which the

different analogues are taken up by the organisms the following experiment was made.

To 40 ml. portions of *Ochromonas malhamensis* culture were added cyanocobalamin, vitamin B₁₂ III, Factor A, Factor B or pseudovitamin B₁₂, each in amounts of 0, 0.01, 0.05, 0.2, 1.0, and 5.0 μ g. The portions of culture were shaken gently for 1 hr. at 29° and then centrifuged. The organisms were extracted and assayed for the different B₁₂-vitamins by using *Lactobacillus leichmannii* and *Escherichia coli* 113-3 as assay organisms (Ford, 1953). The results are shown in Fig. 5.

Because of technical limitations in the assay, the values given for Factor B and pseudovitamin B₁₂ are only approximate. It was quite clear, however, that whereas very little, if any, of Factor B was taken up, the remaining compounds were taken up, and in about the same amount. It would seem then that the inactivity of Factor A and pseudovitamin B₁₂ as growth promoters cannot be attributed to inefficient uptake; the compounds are inherently inactive. Factor B was quite inactive for *Ochromonas malhamensis*, even when it was given together with the 'missing' nucleotide moiety of the cyanocobalamin molecule.

Mechanism of uptake of B₁₂-vitamins

Although several of the B₁₂-vitamins, active and inactive, are taken up by *Ochromonas malhamensis*, it can be shown that cyanocobalamin is taken up preferentially from mixtures containing the inactive analogues. The process seems to be associated with the presence in the organisms of a specific vitamin B₁₂-binding component. Extracts of disrupted *O. malhamensis* have the property of binding cyanocobalamin, vitamin B₁₂ III, Factor A and pseudovitamin B₁₂ in roughly equivalent amount, in the sense of preventing their passage through a cellophan membrane. With these cell extracts, as with intact organisms, cyanocobalamin itself is preferentially bound. Gregory & Holdsworth (1953) reported very similar behaviour of cyanocobalamin in sow's milk and in preparations of 'intrinsic factor'. The property of binding vitamin B₁₂ is typically associated with the gastric secretion in man and in certain higher animals, and is believed to be concerned with uptake of the vitamin. From present indications it may well prove that the mechanism for the uptake of vitamin B₁₂ is essentially the same in *O. malhamensis* as in the higher animals. Certainly *O. malhamensis*, with its animal-like specificity for the vitamin, should prove a most useful model for studies on vitamin B₁₂ metabolism. These observations just outlined about the mechanism of vitamin B₁₂-uptake will be further discussed in a later paper. They are quoted here to help the understanding of experiments (p. 168) on competitive relationships between the B₁₂-vitamins.

Binding of vitamin B₁₂ in culture fluids. In cultures of *Ochromonas malhamensis* the property of binding vitamin B₁₂ is not confined to the organisms; it appears also in the culture fluid during growth. In proportion to the vitamin B₁₂ requirement of the organisms, this extracellular binding capacity is very large.

A culture (400 ml.) of *Ochromonas malhamensis*, grown as described earlier (p. 162) was centrifuged and the supernatant liquid decanted. To 9 ml. portions of this culture liquid were added 1 ml. portions of aqueous solutions containing 0, 0.01, 0.02, 0.05 or 0.1 $\mu\text{g.}$, respectively, of cyanocobalamin. Each sample was then ultrafiltered through Visking cellulose tubing, by the technique described by Gregory (1954), and the ultrafiltrates were tested for cyanocobalamin by means of *Lactobacillus leichmannii*. The results are shown in Table 2.

Table 2. *Binding of cyanocobalamin by Ochromonas malhamensis culture liquid*

Cyanocobalamin added ($\mu\text{g.}/\text{ml.}$ culture liquid)	Cyanocobalamin in ultrafiltrate ($\mu\text{g.}/\text{ml.}$)	Cyanocobalamin 'bound' ($\mu\text{g.}/\text{ml.}$ culture liquid)
0	0	—
0.001	0	0.001
0.002	0	0.002
0.005	0.001	0.004
0.01	0.006	0.004

It is open to question whether the release of 'binding' material into the medium is connected with the mechanism for concentrating vitamin B_{12} from the environment. An analogy with the 'intrinsic factor' in the gastric secretion may be far-fetched, but does at least seem plausible. The degree of binding in the *Ochromonas malhamensis* culture liquid increased with the optical density of the culture, and was not particularly associated with old cultures. But, assuming that the secretion of a vitamin B_{12} -sequestering substance is a characteristic of actively growing organisms, it is difficult to conceive how, except under very restricted conditions of growth, such a substance might be of direct aid in trapping the vitamin. It seems much more likely that it actually inhibits growth. Thus, with *Euglena gracilis* Kristensen (1955) found that the culture liquid contained a thermolabile factor which severely inhibited the growth response to limiting concentrations of cyanocobalamin. And *E. gracilis* culture liquids, like those of *O. malhamensis*, bind vitamin B_{12} . When graded amounts of cyanocobalamin were added to a culture of *O. malhamensis* the efficiency of uptake by the organisms was greater at the higher concentrations. All this suggests that for *O. malhamensis* the cyanocobalamin complex is much less active or readily available than is the free vitamin.

Tests for synergism and antagonism

Effect of pseudovitamin B_{12} on the uptake of cyanocobalamin. Cyanocobalamin (0.02 $\mu\text{g.}$) was added with graded amounts of pseudovitamin B_{12} to 40 ml. portions of a culture of *Ochromonas malhamensis*. These samples were shaken for 2 hr. at 30° and centrifuged. The organisms and supernatant liquids were then separately extracted and assayed for cyanocobalamin by using *O. malhamensis* as assay organism (Ford, 1953). The influence of pseudovitamin B_{12} on the uptake of cyanocobalamin is shown in Fig. 6. At 0.25 $\mu\text{g.}$, pseudo-

vitamin B₁₂ increased the uptake of cyanocobalamin; above this quantity the uptake of cyanocobalamin was progressively inhibited. In other similar experiments, specimens of Factor A, Factor B and 5:6-dimethyl-benziminazole (DMB) were examined for their effects on cyanocobalamin uptake. Factor A behaved like pseudovitamin B₁₂. At low concentrations both compounds increased the uptake of cyanocobalamin, and in higher concentrations both were increasingly inhibitory. Factor A was, however, appreciably less inhibitory than pseudovitamin B₁₂. Factor B had no significant effect on cyano-

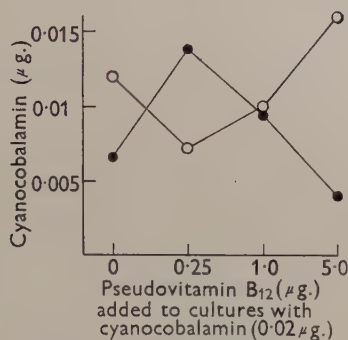


Fig. 6. Effect of pseudovitamin B₁₂ on the uptake of cyanocobalamin. Cyanocobalamin (0.02 μg.) was added to 40 ml. portions of a culture of *Ochromonas malhamensis*, together with graded amounts of pseudovitamin B₁₂. Figure shows distribution of the added cyanocobalamin between cells (●—●) and culture liquors (○—○), after incubation for 2 hr.

cobalamin uptake. DMB, even at 50 μg./ml., had no effect on uptake. DMB, has been shown to inhibit the uptake of cyanocobalamin by *Escherichia coli* (Oginsky & Smith, 1953), and to inhibit competitively the response of *Euglena gracilis* to the vitamin (Hendlin, 1953). DMB is present in the molecule of cyanocobalamin, but not in Factor B. And as Factor B is inactive for *Lactobacillus leichmannii* and very poorly taken up by *O. malhamensis*, it seems possible that the mechanism of uptake involves the linkage of the base (DMB) of the vitamin nucleotide with a receptor protein (or binding factor). The present finding indicates only that the free base does not hinder the uptake of cyanocobalamin by saturation of the binding mechanism.

Effects of pseudovitamin B₁₂ on the rate of growth of Ochromonas malhamensis. Ford (1953) reported that under conditions specified for the assay of vitamin B₁₂ with *O. malhamensis*, pseudovitamin B₁₂ was for practical purposes inactive. Certainly the highest concentrations of pseudovitamin B₁₂ likely to be present in natural materials would have no appreciable influence on the degree of growth measured after incubation for 72 hr. Against this, it is reasonable to infer from the observations so far recorded in this paper that a form of metabolite-antimetabolite relationship should be demonstrable between cyanocobalamin and pseudovitamin B₁₂. Pseudovitamin B₁₂ was tested over a wide range of concentrations, and at three different concentrations of cyanocobalamin, for its effect on the rate of growth of *O. malhamensis*; the results are shown in Fig. 7.

It was found that the lower concentrations of pseudovitamin B₁₂ caused a slight but consistent increase in the growth rate. At higher concentrations of pseudovitamin B₁₂ growth was progressively inhibited and the inhibition was annulled by cyanocobalamin. Other analogues were also tested; Factor A behaved in the same manner as pseudovitamin B₁₂, while Factors B, C and D were quite inert and had no effect on the growth-rate.

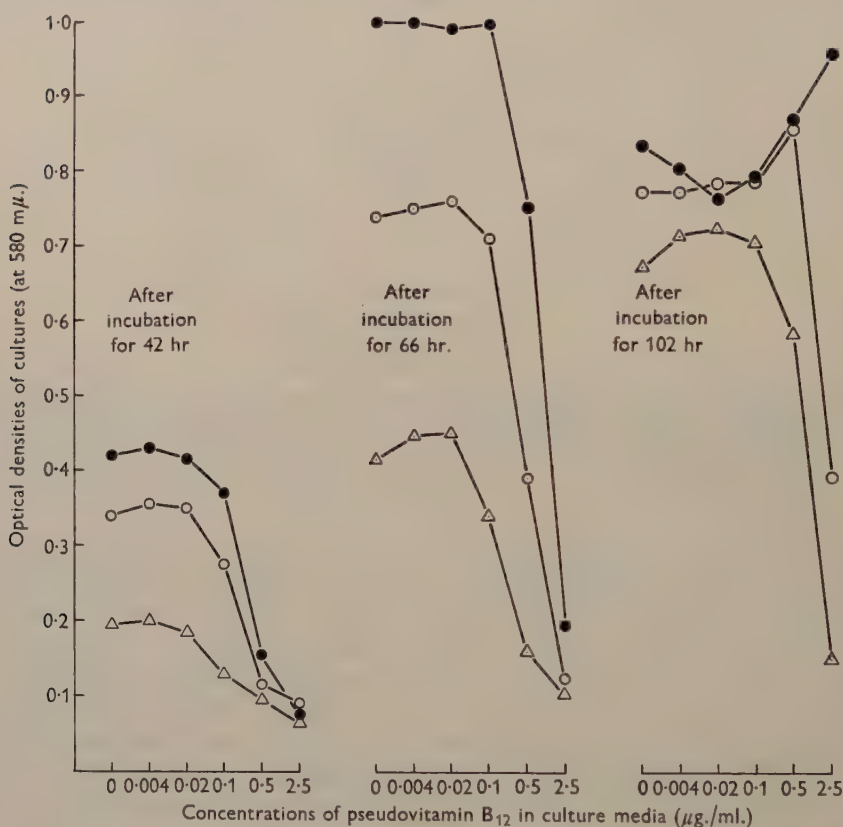


Fig. 7. Influence of pseudovitamin B₁₂ on the rate of growth of *Ochromonas malhamensis* in media containing cyanocobalamin at: (1) 0.0002 µg./ml. (△—△); (2) 0.0005 µg./ml. (○—○); (3) 0.001 µg./ml. (●—●).

Stimulation of growth by the lower concentrations of pseudovitamin B₁₂ and Factor A can be interpreted as a further indication that bound cyanocobalamin is less readily absorbed than the free vitamin (see p. 168). It can be argued that by saturating the binding material in the culture liquid, these analogues would release free cyanocobalamin to the organisms. At higher concentrations the analogues would tend increasingly to inhibit growth by blocking the cell mechanisms for taking up and storing cyanocobalamin.

CONCLUSIONS

The pattern of response to the vitamin B₁₂ group of compounds shown by *Ochromonas malhamensis* seems to be broadly characteristic of a wide range of organisms, from soil bacteria (Ford & Hutner, 1957) to the chick (Coates *et al.* 1956). Many other organisms are less selective and can make use of one or more of the vitamin B₁₂-like compounds. A similar situation is probably found among those bacteria which synthesize vitamin B₁₂: some manufacture several of the B₁₂-vitamins, whereas others seem to produce only the classical vitamin B₁₂. No instance has yet been found of an organism for which any analogue of vitamin B₁₂ is active, and for which cyanocobalamin itself is inactive. Such an organism, if one exists, would be most useful for comparative studies. It seems that the B₁₂-requiring auxotrophs can be differentiated according to whether or not the analogues containing a purine nucleotide are active for them.

Competitive inhibition of cyanocobalamin by pseudovitamin B₁₂ has been reported in a number of soil bacteria (Ford & Hutner, 1957). Further study of one of these bacteria (isolate no. 56 of Lochhead & Burton, 1955) shows that it resembles *Ochromonas malhamensis* in the relation between its rate of growth and the concentration of cyanocobalamin.

There can be no doubt that in the soil (Lochhead & Thexton, 1951), in natural waters (Droop, 1957*a*) and in animals (Ford, Kon & Porter, 1952) the B₁₂-vitamins are of major ecological importance. As an aid to the understanding of the natural situation, studies of models with such organisms as *O. malhamensis* can be of great value.

In the matter of the importance of vitamin B₁₂ in marine productivity, the paramount need seems to be for more information. Little is known of the origins and distribution of marine vitamin B₁₂; even less is known of the relative abundance of the vitamin B₁₂ analogues, although Droop (1957*a*) concluded that these may have an importance equal to that of the vitamin. One can conceive that the evolution of a plankton community might be closely linked with the economy of the B₁₂-vitamins. But whether they are in fact of such over-riding importance in marine ecology is still open to question.

I would like to thank Dr M. R. Droop for his generous help in appraising some of the data presented in this paper. I am grateful also to Drs M. E. Gregory and S. K. Kon for helpful criticism and to Mr D. G. Scardifield for technical assistance.

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Clonal Development of the True Slime Mould, *Didymium nigripes*

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SUMMARY: *Didymium nigripes* has been grown in two-membered cultures with *Aerobacter aerogenes* as its bacterial associate. All phases of the developmental cycle were observed, including spore germination, myxamoeboid growth, plasmodium formation and growth, and fruiting. Both spores and myxamoebae have regularly yielded clonal populations displaying normal development terminating with the appearance of fruits. Efficiency of plating determinations showed that spores and myxamoebae were 100 % viable under the proper conditions. Greatly decreased spore viability was encountered when plasmodia were permitted to fruit in the presence of the metabolic products of their bacterial associate. Plasmodium formation was inhibited by the presence of 2 % (w/v) glucose or 0.2 % (w/v) brucine. However, the myxamoebae grew normally under these conditions.

The members of the Class Myxomycetes, Phylum Myxomycophyta, offer unique opportunities for the study of heterokaryosis and nuclear-cytoplasmic interrelationships. Unlike their relations, the cellular slime moulds (Acrasiales), they have been neglected as experimental organisms largely because of the difficulties encountered in finding strains which can be made to complete their life cycle reproducibly in the laboratory. The life cycle is as follows: the spores germinate liberating flagellate myxamoebae which undergo several divisions before fusing to form plasmodia. The plasmodia develop into large multinucleate masses which enter the stationary growth phase and differentiate into fruiting bodies. During this process meiosis occurs (Wilson & Ross, 1955) and spores are formed. The conditions for spore germination, growth, and for the formation of fruiting bodies have unfortunately remained largely undefined. The complete life cycle has thus been only sporadically observed. It has been possible to surmount the various difficulties of cultivating these organisms in the case of *Didymium nigripes*. The present communication describes the cultural conditions which have permitted reproducible completion of the life cycle in less than 7 days with good spore viability and includes a method for the isolation and growth of clones.

RESULTS

Growth of mass cultures

Spores of *Didymium nigripes* were suspended by crushing fruiting bodies against the sides and bottom of a conical centrifuge tube which contained 1 ml. water. When the suspension was bubbled vigorously with air the stalks, clumps, and pieces of debris remained on the sides of the tube leaving a

homogeneous spore suspension. A sample of this suspension was spread together with a few drops of a 48 hr. *Aerobacter aerogenes* culture on SM/5 medium (2 g. Bacto-peptone; 2 g. glucose; 0.2 g. yeast extract; 0.2 g. K_2HPO_4 ; 0.3 g. KH_2PO_4 ; 0.2 g. $MgSO_4 \cdot 7H_2O$; 20 g. agar; in 1 l. distilled water; final pH value 6.0-6.3). The plates were incubated at 22°. Spores germinated during the next 7 hr. to yield myxamoebae which soon developed flagella. Plasmodia appeared after 3 days, and continued to grow for 2 days until their bacterial food supply was exhausted. Fruiting bodies were then formed within 48 hr.

Prolongation of the amoeboid stage

The addition of 2% (w/v) glucose or 0.2 (w/v) brucine to the SM/5 medium inhibited plasmodium formation. Under these conditions spore germination and myxamoeboid growth took place as usual, but, instead of forming plasmodia, the amoebae continued to multiply until the plate was cleared of bacteria. When these amoebae were subcultured on similar media, they repeated this process. When subcultured to SM/5 medium they formed plasmodia which completed their life cycle normally. Sulphanilamide (0.1%, w/v) delayed plasmodium formation for 24 hr. when added to SM/5 medium but did not affect the growth of the myxamoebae.

Isolation of clones

Clones were obtained by using the method of Sussman (1951) originally devised to permit clonal growth of the cellular slime moulds, the Acrasiales. Samples containing up to fifty spores of myxamoebae were spread with *Aerobacter aerogenes* on SM/5 medium. Four days later small clear areas (plaques) containing myxamoebae appeared in the film of bacterial growth. These myxamoebae soon formed plasmodia which grew as a ring around the edge of the plaque and slowly enlarged it while feeding on the bacterial layer (Pl. 1, figs. 1-4). When the clones were about 1 cm. in diameter they were transferred along with the underlying agar to PA medium (0.2 g. K_2HPO_4 ; 0.3 g. KH_2PO_4 ; 20 g. agar; in 1 l. distilled water; final pH value 6.0-6.3) and were allowed to fruit. Of eighteen clones thus tested, all fruited normally to yield viable spores which in turn produced clones which completed the life cycle in the usual manner. A single clone was selected for the remainder of the work reported here.

Efficiency of plating

A single empty spore case was often observed in a plaque (Pl. 1, figs. 2, 3) indicating that a single spore had given rise to the clone. It was necessary to determine, however, the proportion of spores which were viable and could produce normal plasmodia. This was accomplished by determining the ratio of spores plated to plaques formed (efficiency of plating, expressed as %). Large plasmodia were transferred to PA medium and allowed to fruit. Two days later the fruits were picked, and the spores homogeneously suspended and counted in a Levy haemocytometer. The spore suspension was diluted appropriately and replicate samples containing up to fifty spores were spread,

together with *Aerobacter aerogenes*, on SM/5 medium. Four days later clones appeared and were counted; the efficiency of plating of such spores approached 100%. The degree of germination was also determined by observing microscopically the ratio of empty spore cases to total spores counted. Such observations yielded results which confirmed those obtained by the plating method. Spore viability remained near 100% for a month after fruiting. Myxamoebae when washed off plates of 2% (w/v) glucose + SM/5 medium and plated in a similar manner, also had an efficiency of plating near 100%.

When plasmodia were allowed to continue growing and to form fruiting bodies on the same plate which had supported their earlier growth, the viability of the resulting spores was exceedingly variable. The most likely cause of this irregularity seemed to be that metabolic products of the bacteria affected spore viability. This hypothesis was tested by scoring the viability of spores originating from plates which had been exposed to bacteria for varying periods of time. Sixteen plates of SM/5 medium were spread with *Aerobacter aerogenes*. Each day for 8 days two of these plates and a PA medium control plate were inoculated with plasmodia. Determinations of efficiency of plating were made on spores from each plate 2 days after the completion of fruiting. It can be seen from the results given in Table 1 that spore viability fell to zero after seven days of pre-incubation of the bacteria.

Table 1. *The effect of ageing of Aerobacter aerogenes on viability of spores of Didymium nigripes*

Spores in the experimental series were from plasmodia which had fruited on SM/5 medium after having been exposed to bacteria for varying periods of time. Control spores were from plasmodia which fruited on PA medium. EOP=efficiency of plating.

Days of pre-incuba- tion of <i>A. aerogenes</i>	Experimental				Control	
	1		2			
	No. of plaques	EOP (%)	No. of plaques	EOP (%)	No. of plaques	EOP (%)
0	38 ± 8	75	56 ± 9	112	45 ± 3	90
1	32 ± 2	65	—	—	40 ± 3	80
2	36 ± 6	72	52 ± 3	104	—	—
3	22 ± 7	44	22 ± 6	44	49 ± 2	98
4	12 ± 8	24	17 ± 5	34	53 ± 11	106
5	23 ± 2	46	—	—	43 ± 3	86
6	23 ± 2	46	6 ± 5	12	53 ± 5	106
7	0	0	0	0	54 ± 7	108

DISCUSSION

Brucine and glucose can conceivably act at either of two stages in the developmental cycle. These agents may either prevent the fusion of myxamoebae to form plasmodia, or, alternatively, having permitted fusion to occur, they may prevent the usual subsequent enlargement of the plasmodia. Against the latter possibility it should be noted that even newly formed plasmodia are readily distinguishable from myxamoebae on the basis of size, granulation and

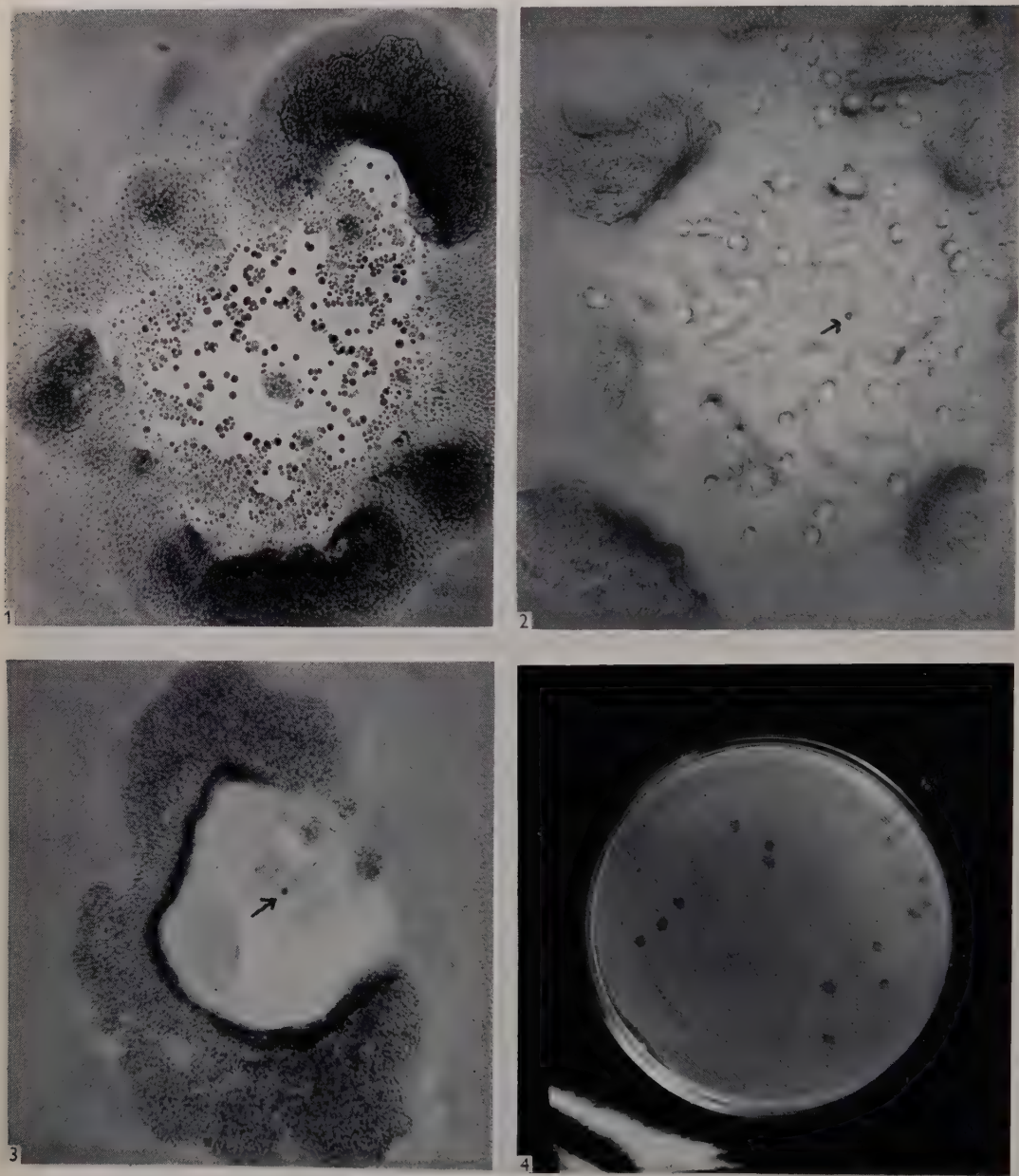
protoplasmic streaming. Thus it is reasonable to expect that they would have been detected on brucine or glucose plates had they been present. It would appear, therefore, that the first explanation can be tentatively accepted until such time as definitive studies with genetically marked stocks can be performed. The retention of the amoeboid stage by glucose or brucine has opened many interesting subjects to investigation because it is now possible to obtain myxamoebae in almost limitless quantities. Thus a quantitative analysis of the kinetics of plasmodium formation is practicable and is now being made. While it is difficult to measure accurately the growth of a plasmodium, it is easy to count an increase in the number of myxamoebae; their use should therefore simplify nutritional studies.

Spore viability. Cadman (1931) and Parker (1946) did not succeed in germinating single spores of *Didymium*; they postulated that interactions between spores were necessary for germination. There can be no doubt that isolated spores of our strain are capable of germination. When fifty spores are spread per plate the distance between plaques is often a centimetre. Interactions would not be likely to operate over this distance during the 7 hr. between plating and the end of germination. Because all spores are capable of developing singly our strain must be homothallic. The possibility that mating type is segregated during the first divisions after spore germination was eliminated when plasmodia and fruits were produced by clones derived from single amoebae.

The difficulty of obtaining viable spores is perhaps the principal factor which heretofore has discouraged laboratory cultivation of *Myxomycetes*. Although it is dangerous to generalize about a group as diverse as this one, the experiments reported here indicate that this difficulty may have been due to the use of spores which had been poisoned by metabolic products of the bacterial associate. Spores which regularly have a high degree of viability can be obtained in two ways. Either large plasmodia can be transferred to PA medium and be allowed to fruit, or, when large plasmodia are not available, plasmodia can be placed on SM/5 medium plates newly spread with bacteria. Because under the latter conditions the plates are inoculated with plasmodia instead of spores, fruiting occurs within 3 days rather than after 7, and is therefore not affected by metabolic products of the bacterial associate (see Table 1).

We wish to thank Dr A. L. Cohen who kindly sent us the strain of *Didymium nigripes* used in this investigation.

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N. S. KERR & M. SUSSMAN—*DIDYMIUM NIGRIPES*. PLATE 1

(Facing p. 177)

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EXPLANATION OF PLATE

- Fig. 1. A young plaque. Myxamoebae, small plasmodia, and larger plasmodia are visible. ($\times 65$.)
- Fig. 2. A plaque showing small plasmodia and larger plasmodia. The spore case from which the clone arose is indicated by the arrow. ($\times 110$.)
- Fig. 3. A mature plaque. Most of the small plasmodia had fused into one large one which then fed on the bacteria at the edge of the plaque. The spore case from which the clone originated is indicated by the arrow. ($\times 65$.)
- Fig. 4. A plate showing 19 clones. These plaques were slightly older than the one shown in figure 3. ($\times \frac{1}{3}$.)

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Infectivity Titrations and Particle Counts of Adenovirus Type 5

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SUMMARY: Infectivity titrations and electron microscopic spray counts were performed on purified preparations of adenovirus type 5. The number of virus particles/50 % infective units varied from 10 to 100 with different preparations.

Infectivity titrations of adenoviruses are usually based on their cytopathic effect in cultures of HeLa cells and end-points, calculated from all-or-none responses, are given as the dilution of the preparation under test which gives a 50 % response. It has been shown (Pereira & Kelly, 1957) that the dose-response curve of the infective action of adenovirus type 5 in HeLa cells is consistent with the hypothesis of independent action of infective units, a single unit being sufficient to initiate infection. The present investigation was designed to determine the relation between these infective units and electron microscope counts of the numbers of virus particles.

METHODS

Virus strain. The prototype strain of adenovirus type 5 was received from Dr R. J. Huebner (National Institutes of Health, Bethesda, Maryland, U.S.A.) and propagated in HeLa cell cultures. The materials used in the present study consisted of pooled cells from cultures showing advanced cytopathic effect. Each pool contained cells either from ten bottles (each with about 10^7 cells at the time of inoculation) or from 200 to 300 tubes used in routine titrations of the strain under study.

Virus purification. The method used was based on the technique described by Gessler, Bender & Parkinson (1956). Pooled cells were washed with 25 ml. distilled water and centrifuged at 600 g for 10 min. The supernatant fluid was discarded and the packed cells resuspended in 2 ml. of M/15 phosphate buffer (pH 7.3). This suspension was added to 4 ml. of Arcton 63 (Imperial Chemical Industries, London, S.W. 1) and homogenized in an M.S.E. universal container blender for 2 min. at maximum speed. The homogenate was centrifuged for 10 min. at 600 g and the aqueous fraction decanted and saved. Another 2 ml. volume of phosphate buffer was added to the cell + Arcton fraction and followed by homogenization and centrifugation as above. This process was repeated a third time and the three aqueous fractions were pooled. The material obtained was opalescent, and when examined in the electron microscope was seen to contain a considerable amount of amorphous debris in addition to the virus particles. Further purification was achieved by submitting this material to several cycles of freezing at -10° and thawing at 37° .

At each cycle a precipitate formed and this was separated by centrifugation at 1000 *g* for 10 min., without measurable loss of virus activity. Three or four cycles of freezing and thawing were usually sufficient to yield almost water-clear preparations suitable for spray counts in the electron microscope (see Pl. 1).

Virus titrations. These were performed in cultures of HeLa cells propagated and maintained by methods previously described (Pereira & Kelly, 1957). Virus dilutions were made in broth saline (10% tryptic digest broth in normal saline) at 0.5 log steps, using a fresh pipette for each dilution. Volumes of 0.1 ml. of appropriate dilutions were inoculated into each of 4 cultures of HeLa cells which were incubated without rotation at 37° for 27 days, with 3 fluid changes at intervals of 6 to 7 days. The 50% end-point was obtained by the method of Reed & Muench (1938), and recorded as the number of 50% tissue culture doses (TCD₅₀)/ml. The standard error of each titration was calculated according to Pizzi's (1950) formula.

Table 1. *Particle counts and infectivity titres of adenoviruses type 5*

Material	Particles/ml. ($\times 10^{10}$)	Infective doses/ ml. ($\times 10^{10}$)	Particles/ infective dose
H554-c	6.5	0.10	65
H554	6.8	0.68	10
H565	37.4	0.46*	82
H566	20.7	0.20*	103

* From geometric mean of two titrations.

Particle counts. The purified virus preparations were mixed with equal volumes of an aqueous suspension of polystyrene latex spheres of diameter 0.16 μ (Dow Chemical Company: Lot no. LS 055 A). The latex suspension had a count of 1.0×10^{11} particles/ml. as determined from its dry weight, the known density (1.05 g./cm.³) of the spheres and their diameter which was measured in terms of the spacing of an aluminium replica of a standard ruling. Bovine albumin was added to the mixture (0.05%, w/v) which was then sprayed in the usual way (Williams & Backus, 1949; Backus & Williams, 1950) on to carbon films carried on electron microscope specimen supports. These were lightly shadowed with palladium and photographs were taken of the micro-droplets at a magnification of $\times 7,000$. The virus particles could be clearly identified as electron-dense spheres of *c.* 70 $m\mu$. diameter and the ratio of the count of these particles to the count of the latex particles was determined. The counts were checked for randomness and statistical significance by the method given by Luria, Williams & Backus (1951).

RESULTS

Infectivity titres and particle counts obtained from four virus preparations are recorded in Table 1. The standard errors of the particle counts varied between 10 and 20% and those of the infectivity titrations between 0.18 and 0.30 log₁₀ units.

The mean number of virus particles/infective dose ranged from 10 to 103. This variation may be due to differences in sensitivity of separate batches of HeLa cell cultures. It is also possible that the different preparations used contained varying proportions of inactive virus particles, as no special precautions were taken to insure maximum infectivity of the starting materials. Preliminary experiments had shown that infectivity titres of adenovirus type 5 were not significantly decreased by the Arcion 63 treatment described above.

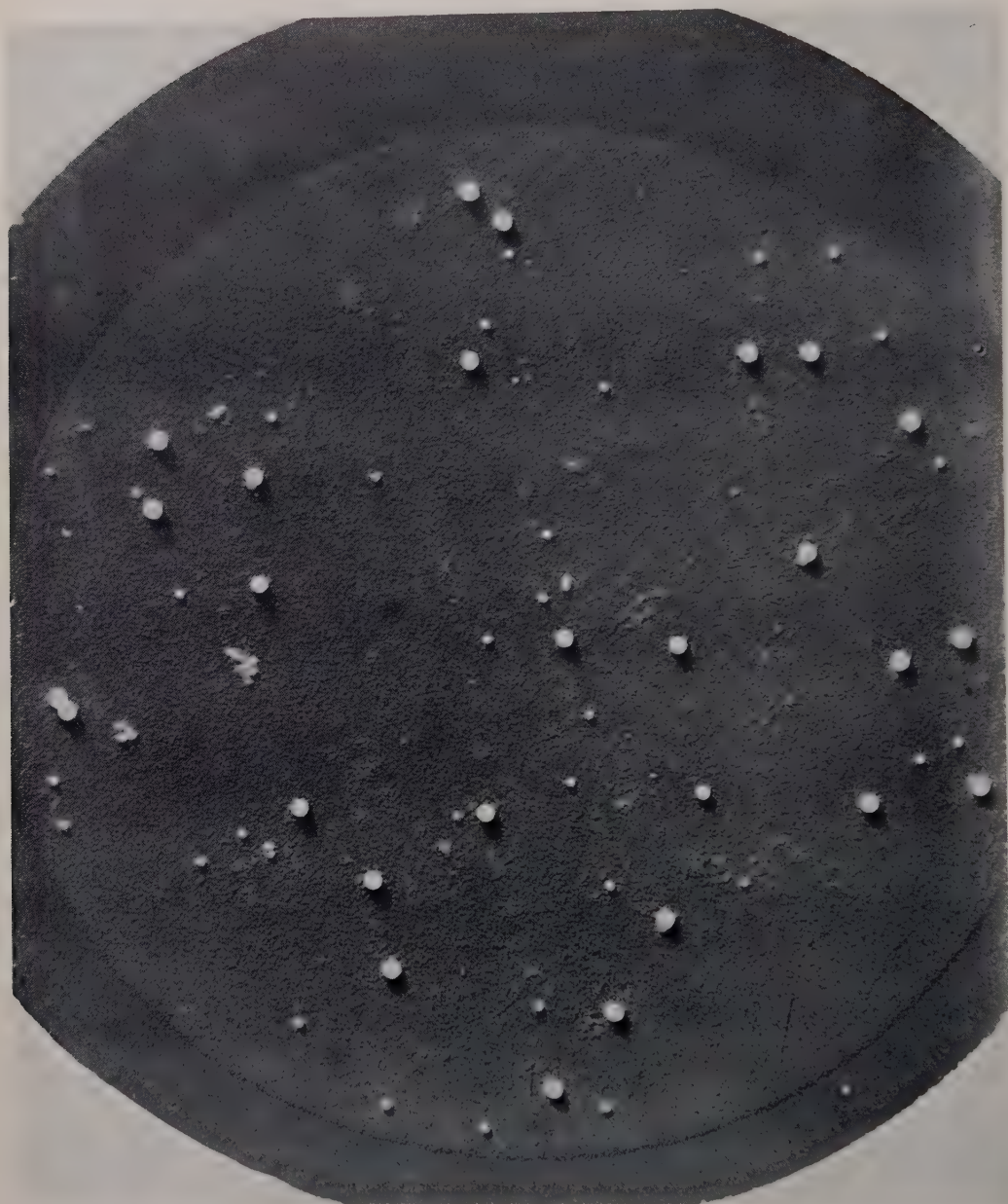
COMMENTS

The numbers of virus particles/infective dose obtained in the present study are very similar to the corresponding values found for other animal viruses (Isaacs, 1957; Schwerdt & Fogh, 1957; Dumbell, Downie & Valentine, 1957). They differ, however, from the ratio of 10^4 particles/TCD50 of adenovirus type 4, reported by Tousimis & Hilleman (quoted by Tousimis & Hilleman, 1957). This discrepancy is rather too large to be due to differences in the properties of the two virus types and is probably accounted for by the short period of incubation (3 days) of the test cultures in the titration technique used by Tousimis & Hilleman. The sensitivity of adenovirus titrations in HeLa cells varies greatly with the length of this period of incubation and maximum titres are reached only after incubation for 3–4 weeks (Pereira, unpublished).

We wish to thank Mr D. J. McGillicuddy for valuable technical help.

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H. G. PEREIRA & R. C. VALENTINE—ADENOVIRUS PARTICLE COUNTS. PLATE 1

(Facing p. 181)

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EXPLANATION OF PLATE

Electron micrograph of a typical spray droplet used in making the virus particle count. The larger spheres are the latex indicator particles ($0.16\ \mu$ diameter) and the smaller dense objects ($c. 70\ m\mu$ diameter) the adenovirus particles. The droplets were metal shadowed with palladium. $\times 16,000$.

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The Biology of *Harposporium anguillulae*

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SUMMARY: *Harposporium anguillulae* Lohde was isolated in pure culture from an infected nematode and its behaviour on culture media under various conditions is described. The life history of the fungus in the nematode host has been investigated and the infection traced back to germinating conidiospores in the oesophagus. Two other *Harposporium* spp. were also isolated and it is shown that their mode of infection is similar to the one described for *H. anguillulae*. Because of these findings a new theory for the mode of infection is proposed and its significance in the ecology of the fungus discussed.

Among the predacious fungi which attack nematodes in soil, certain *Arthrobotrys* and *Dactylella* spp. have received much attention. These fungi can easily be studied in the laboratory since they grow well on simple nutrient agar media and their interesting mode of catching nematodes by means of sticky or constricting loops can be directly observed under the microscope. They have also been tested in experimental field studies as to their effectiveness in the biological control of certain root eelworms (Duddington, 1957). By contrast, no experimental work has been done so far with those fungi which infect nematodes by means of specialized conidiospores and kill them through the endozoic mycelium which develops from these spores within the host's body cavity. This is all the more surprising, as these fungi are not uncommon in soil and their parasitic relationship with soil nematodes was recognized long ago (Lohde, 1874).

A number of mycelial fungi with infective spores, most of them belonging to the genus *Harposporium*, have been described (Karling, 1938; Drechsler, 1941; Shepherd, 1955), but as far as we know, none of these has ever been studied in pure culture, and such fundamental questions as the mechanism of infection or the specificity of the parasite for the various soil eelworms still remain open. The present investigation, based on a detailed biological study of three *Harposporium* spp. is a contribution to this discussion.

METHODS

The strain of *Harposporium* used in the investigation was found in a sample of soil and manure which had served as substrate for the cultivation of mushrooms at Matzuba (northern Israel). A sample (0.1 g.) of this soil had been incubated at a temperature of 28-30° on a plate of tap-water agar, consisting of agar-agar strips (2%, w/v) in tap-water. After an incubation period of 14 days dead eelworms were seen on the agar surface, and septate hyphae with typically arcuate spores were seen sprouting from their bodies, indicating an infection with *Harposporium* sp. (Pl. 1, fig. 1).

Our fungus closely resembles *Harposporium anguillulae* Lohde as described by Karling (1938), and although there are some slight differences in the

measurements of the conidiospores (width 1μ , length $5-8\mu$ in our fungus as against 1.5μ and $5-14\mu$, respectively, in Karling's description) we do not feel justified in creating a new species for our organism. A complete identification will be possible only when strains of this parasitic fungus are compared on identical culture media or in the same species of host organism. As far as we know this has not been done yet.

The eelworms used in our experiments included several of the rhabditic species of nematode and one *Dorylaimus* sp. which had previously appeared in various soil cultures. None of these species was identified. The eelworm cultures were kept on tap-water agar at room temperature and *Escherichia coli* was added as food organism. Handling of the eelworms was facilitated by cutting holes of about 1 cm.^2 surface, so called lagoons, into the agar layer. These holes were filled with water, and large numbers of eelworms accumulated there and became infected when conidiospores of any of the examined *Harposporium* spp. were added to the water. For the microscopic examination of infections in living eelworms the following technique was developed. A drop of fluid from a lagoon with infected eelworms was mixed on a slide with a small amount (less than a loopful) of a 1% (w/v) aqueous solution of erythrosin. This preparation was then warmed gently by placing the slide without coverslip near an electric lamp until the edge of the drop began to dry. It was then covered with a coverslip and dried for 1 hr. more at room temperature until the coverslip exerted a slight pressure on the eelworms in the drop. The clearest pictures were usually obtained just before the preparation dried up entirely or the pressure of the coverslip burst the cuticle of the eelworm. The erythrosin stained the external hyphae and conidiospores of the *Harposporium* spp. and also had an anaesthetic effect on the movements of the eelworms.

RESULTS

Isolation of the infecting fungus

One infected eelworm was cut out of the agar medium, together with the small piece of agar adhering underneath and transferred to another plate which besides tap-water agar contained 100 p.p.m. penicillin and the same amount of streptomycin to suppress development of bacteria as far as possible; c. 0.1% (w/v) of 'Dithane' (a commercial preparation of zinc ethylene-bis-dithiocarbamate made by Rohm and Haas) was also added. This substance has a fungistatic action on many soil fungi but apparently not on a *Harposporium* sp. growing inside a nematode. After 2 days a small piece (about 0.25 cm.^2) of yeast-extract agar was cut from an agar plate and placed near the explanted nematode. The composition of the yeast medium was (g.): peptone, 0.5; glucose, 2; yeast extract (Difco), 0.2; agar-agar, 2; 100 ml. distilled water.

The growth of the fungus was enhanced by the nutritive material diffusing from the yeast-extract agar but the concentration was not high enough to support luxuriant growth of contaminating organisms which were still present on the surface of the eelworm. When the hyphae of the *Harposporium* sp. had spread somewhat and penetrated into the solid medium another small agar

block was cut out aseptically from the periphery of this growth and transferred to fresh yeast-extract agar. The transferred piece proved to be free from other micro-organisms and the excised hyphae continued to grow on this medium.

After about 14 days the growth was visible macroscopically as a dense snow-white dome-shaped colony, c. 0.5 cm. in diameter. No conidiospores were found in this culture and the only indication that our isolate was identical with the fungus in the eelworm was the appearance of 'endozoic mycelium' in the innermost part of the colony. This means that the mycelium in the colony looked like the growth inside an infected eelworm, and was composed of two morphologically different components: (a) strands of large thick-walled cells, comparable to the endozoic mycelium, in the centre of the colony; (b) thin-walled aerial hyphae in its outer parts. Sometimes the thick-walled mycelium showed a light green coloration. Subsequent cultures which have been grown now for several months on artificial media, showed a more luxuriant growth which covered the agar surface with a fluffy spreading mycelium.

In later experiments the method used for the isolation of strains of *Harposporium* was much simpler. A few drops from a lagoon with infected eelworms were spread over a plate of glucose yeast-extract agar containing about 0.1 mg./ml. of Aureomycin. The hyphae which emerged from the infected eelworms grew well on this medium while most bacteria were inhibited. After 3-4 days the small areas of mycelium which had formed around the infected eelworms were examined microscopically on the plate and when found to be free from contamination were cut out from the agar and transferred to a glucose yeast-extract agar slope.

Formation of conidiospores in cultures

The lack of conidiospores in these cultures made them unsuitable for experiments on the infection of eelworms. This difficulty was overcome in the following way. Colonies from glucose yeast-extract agar, together with some adhering agar medium, were cut into very small pieces (the size of a pinhead or smaller) and placed on tap-water agar plates; the intention was to imitate more closely the conditions of limited food supply which confront the fungus colony in nature after food material in the invaded eelworm has been used up. The validity of this principle has not been proven but the procedure worked satisfactorily and the fragmented mycelia developed hyphae which, with regard to spore formation, were identical with those observed in natural infections.

Growth requirements of the Harposporium sp.

Although *Harposporium anguillulae* in nature acts like an obligate parasite (at least it has never been reported to occur outside its nematode hosts), it does not seem to have special food requirements. Our organism was able to develop not only on the glucose yeast-extract agar medium mentioned above, but also on a simple glucose mineral-salts medium consisting of (g.): glucose, 1; $(\text{NH}_4)_2\text{SO}_4$, 0.2; K_2HPO_4 , 0.1; MgSO_4 , (anhydrous), 0.5; agar-agar, 2; 100 ml. distilled water. Growth on this medium, however, was much slower and less luxuriant than on the yeast-extract agar.

Our fungus grew on the surface of liquid yeast-extract medium and when this was agitated, as tiny pellets throughout the whole volume of fluid. The conditions in liquid media did not seem to be conducive to the formation of conidiospores; however, some were formed on those colonies which grew attached to the vessel-wall at the boundary between air and liquid.

Germination of the conidiospores

No germinating spores were found on the media commonly used for the detection of *Harposporium* spp. These media have to be low in food content; otherwise bacteria which always occur in the same environment would overgrow the more slowly growing *Harposporium* sp. This difficulty does not arise on nutrient media in which the development of bacteria is selectively inhibited. On glucose yeast-extract agar with 100 p.p.m. aureomycin some spores germinated and the process was followed under the microscope. The germination bud appeared always in the thickest part of the spore on the outer circumference of the crescent. It remained small and thin (less than 1μ in width) but the mycelium which developed without any transition from this tiny outgrowth had the appearance and dimensions of a normal-sized hyphomycete. The germination bud remained as a short neck-like piece between spore and mycelium. The direction of growth of the germ tube was opposite to that of the pointed horns of the spore (Pl. 1, figs. 3-5).

Not all the spores germinated under these conditions, many of them remained dormant even during an observation period of 2 weeks. Germination of spores occurred also on glucose mineral salts medium but, in accordance with the less favourable growth properties of this medium, the proportion of spores which germinated on this medium was rather small.

The penetration of the spore into the body cavity of the eelworm

Several opinions have been expressed about the manner of infection of the host eelworm by *Harposporium* spp. Drechsler (1941) for *H. oxycoracum* described crescent-shaped spores with a drop of sticky fluid adhering to one of the pointed tips. According to his view this fluid served to glue the spore to the outer integument of the eelworm, whence after germination the germ tube entered through the cuticle into the body cavity of the host. Duddington (1957) expressed the opinion that the conidia in *H. anguillulae* and most other species of *Harposporium* were sticky and that with all of them the mode of infection resembled that suggested by Drechsler for *H. oxycoracum*. Duddington rejected the possibility that such spores could enter the host by an oral route because of their too great size. Other authors (Zopf, 1888; Kostka, 1927) have assumed a connexion between spore shape and mode of infection. According to their view the spore pierced the outer cuticle of the nematode mechanically by means of the sharply pointed tips, the force required for this feat being provided by the momentum of the actively moving eelworm itself.

We attempted to decide the question of mode of entry by examining the earliest stages of infection. Hyphae which protruded through the cuticle of the eelworm always indicated a well-advanced infection, with the endozoic

mycelium occupying practically the whole of the body cavity. This stage was obviously too late to supply information about the mode of entry of the parasite. Consequently, we examined eelworms which had come in contact with spores but which were still living and without any external signs of infection. By using the microscopical technique described earlier it was possible to demonstrate the presence of germinating conidiospores inside the body of the eelworm. Such spores were invariably situated in the upper region of the digestive tract, between the oral opening of the buccal cavity and the median bulb of the oesophagus. They appeared to be firmly wedged in this narrow channel with the central part of the spore in close contact with the lining of the tract. The place of contact was also the place of germination of the spore and at the same time the place of entry of the fungus into the body cavity of the eelworm, with the tiny germination bud acting as the means of piercing the wall of the oesophagus. No incipient infection was ever found in or on any other part of the eelworm body, but the oesophagus contained sometimes more than one spore.

The picture of the early stages of infection with this fungus was identical in all the cases observed. The germ tube was situated in or near the muscle tissue of the oesophagus, growing towards the cardiac bulb (Pl. 1, figs. 6-8). After reaching this organ and encircling it, it usually grew in the direction of the head (Pl. 1, fig. 9). At these stages of infection the eelworm was still alive. Very soon afterwards side-branches pushed out from the encircling loop and entered the body-cavity of the host eelworm in the intestinal region. These branches completely dissolved the intestinal tissues, causing death of the host. The fungal growth inside the eelworm body then changed into the morphologically differentiated endozoic mycelium which finally formed typical chlamydospores (Pl. 1, figs. 2, 10).

The hyphae which sprouted from this mycelium pierced the cuticle and later gave rise to sickle-shaped conidiospores. At the point where hyphae protruded through the cuticle they were usually surrounded by a collar-like formation which somewhat resembled a hair socket in an insect cuticle. These sockets were clearly visible in stained preparations or in old infections after disintegration of the hyphae (Pl. 1, fig. 10). It was observed that with this species of *Harposporium* the piercing of the outer cuticle usually started in the head region and that formation of conidiospores was earlier on the hyphae in this region than elsewhere. It was indeed this observation which induced us to search for the start of infection in the upper region of the digestive tract.

Infectivity of the Harposporium strain for diverse species of eelworm

Among the numerous species of eelworms in the soil a large number belongs to the so-called *Rhabditis* group of nematodes. These eelworms feed on bacteria and other particulate matter which they take into the buccal cavity from the surrounding fluid and swallow as solid particles. Other eelworms, and among them many plant-parasites, are unable to swallow particulate food. They suck instead the fluid content of various living cells after piercing the cell wall by means of a protractile stylet in their mouth cavity. A genus of free-

living eelworms in this group is *Dorylaimus*. We had no difficulty in infecting rhabditic eelworms with our fungus. Several species were tested and all gave the same positive result. However, a species of stylet-bearing eelworm belonging to the genus *Dorylaimus*, when tested in the same way, remained free from infection during the whole period of observation, which lasted one month.

Comparison of Harposporium anguillulae with two other Harposporium spp.

While investigating the mode of infection of *Harposporium anguillulae* we isolated from eelworms found in garden soil (Haifa) two additional species of *Harposporium*. One of these organisms produced sickle-shaped spores which were considerably larger than those of *H. anguillulae*. They resembled spores of *H. helicoides* in their dimensions but lacked the mucus droplet described for this species. It was not possible for us, owing to lack of literature, to determine whether this species had already been described or not. We therefore called it, provisionally, species A (Pl. 2, figs. 11, 12). The spores of the other species had the form of short and rather small rods which were provided at their ends with two short thornlike processes. These processes were sharply bent at right angles to the long axis of the spore and pointed in opposite directions. A similar shape has been described for *H. diceraeum* but our strain differed in some details from this species and could not be identified with it. We have called it provisionally, species B (Pl. 2, figs. 15, 16).

The infection of eelworms with species A presented a picture very similar to that observed in *Harposporium anguillulae*. Germinating spores were found in the lumen of the oesophagus and, again, in its upper region only. They were even easier to detect than the spores of *H. anguillulae* on account of their larger size. Occasionally double infections in the oesophagus were seen and in one case three spores were present in this organ. They were arranged in tandem fashion and covered practically the whole section from oral opening to median bulb. The development of the endozoic mycelium of this fungus differed from that characteristic for *H. anguillulae*. Instead of growing in close attachment to the musculature of the oesophagus the hyphae started their growth at some distance from this organ. This was due to a peculiar modification of the germination bud in this species. This bud grew as a fine filament (length 5–6 μ) which penetrated not only the intima of the oesophagus but the entire musculature of this organ. Thus the growth of regular hyphae started directly in the body cavity of the eelworm (Pl. 2, fig. 13). The filament-like germination bud was clearly discernible also when spores germinated on nutrient media (Pl. 2, figs. 19, 20).

Spores of species B in a very similar way developed, on nutrient media, a germination filament before the growth of regular hyphae started, but in the eelworm not a single infection among a large number examined could be traced back to a spore in the oesophagus. Spores were found only in the buccal cavity. This finding was rather surprising as the short and relatively small spores seemed to be much more suitable to enter the oesophagus than the large and unwieldy sickle spores of the other two species (Pl. 2, figs. 14, 17, 18). When the oral cavity of the eelworm was filled with mucus or food-debris it was sometimes difficult to detect the spores there. In these cases diagnosis

could be made by demonstrating the germination filament which originated in that locality and passed into the initial hypha. Species B grew less luxuriantly on glucose yeast-extract agar than the other two species of *Harposporium* but produced many conidiospores on this medium.

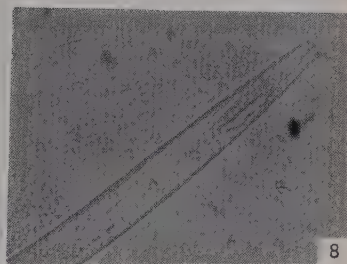
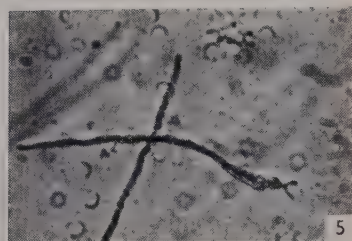
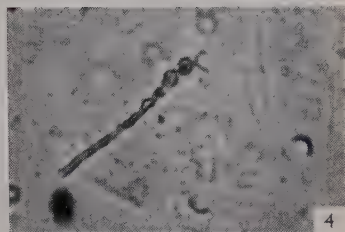
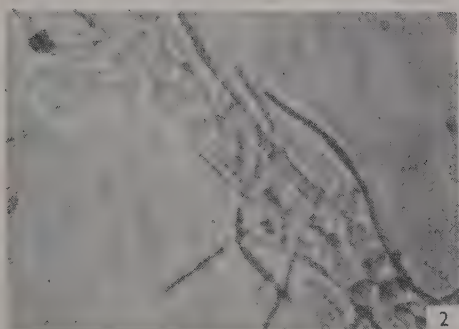
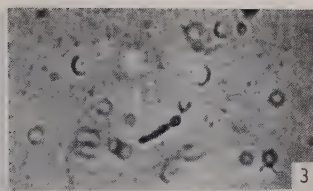
Species A and B, in contrast to *Harposporium anguillulae*, did not form rounded chlamydospores in the infected eelworm, and their external hyphae pierced the cuticula of the eelworm in the head and tail region at practically the same time. All three species of *Harposporium* were able to infect eelworms after prolonged growth on artificial media.

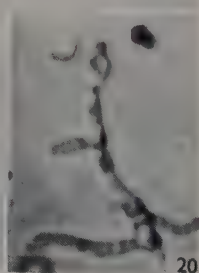
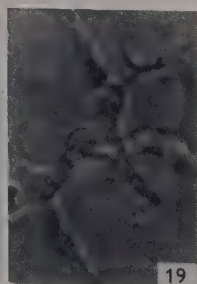
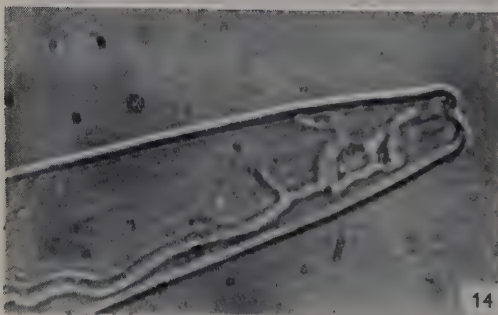
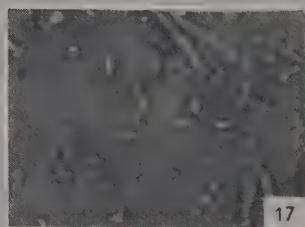
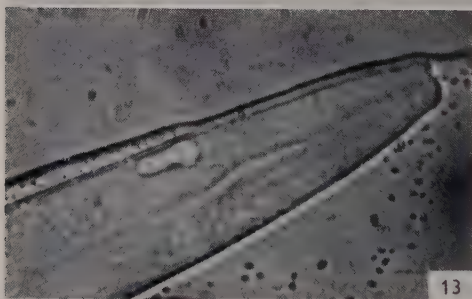
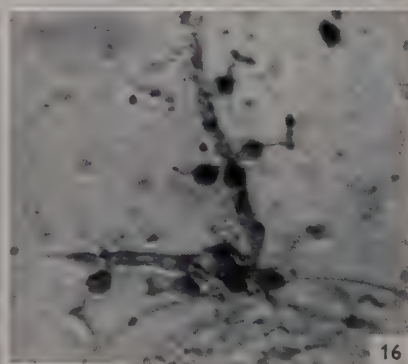
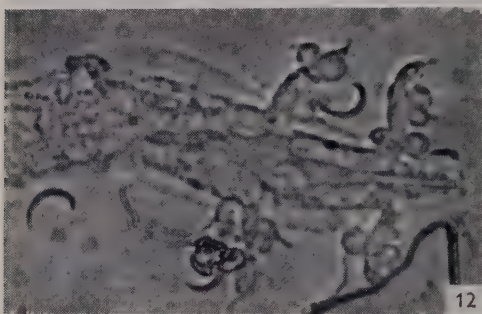
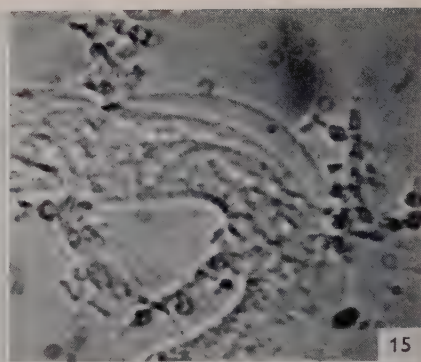
DISCUSSION

The conidiospores of *Harposporium anguillulae* and species A appear to enter the eelworm host by the oral route; otherwise their presence in the buccal cavity or oesophagus is difficult to account for. We must assume that they are taken up and swallowed by the eelworm, although their overall dimensions are larger than the lumen of the buccal cavity or the oesophagus. This would of course be impossible in the case of a straight and rigid channel, it seems however, that the eelworm can bend or twist its alimentary tract in the head region according to the (arcuate or helicoidal) shape of the particle which is to be swallowed. The *Harposporium* spores being very slender can thus be taken up whenever they enter the oral opening with one of their tips. It is not known what induces the spores to adhere to the upper region of the oesophagus. With their crescent-like curvature and pointed tips they resemble a fishhook, and their fixation in the oesophagus may be due to the hooklike function of one or both of these tips. It has, however, not been demonstrated that the oesophagus is actually pierced in that way.

The conidiospores of species B also enter the host by the oral route, but are found only in the buccal cavity and not in the oesophagus of infected eelworms. This may be due, obviously, either to a barrier at the entrance of the oesophagus which prevents spores of this shape to pass, or to inability of these spores to affix themselves in the lumen of this organ once they have entered it. In this case they are probably swept, together with the food, into the stomach to be digested there.

No signs were found which indicated with any of our fungi an invasion of the eelworm through the outer integument. We never observed spores adhering to a living nematode, not even after it had crawled through heaps of detached spores or had brushed against a spore-bearing hypha: furthermore, as mentioned already, incipient infections were seen only in the head region of eelworms. It may therefore be claimed that, with regard to the *Harposporium* spp. studied, infection of eelworms proceeds only through the oral route. The experiment with the *Dorylaimus* eelworm supports this claim: if the swallowing of a conidiospore is essential in order that host be infected with the fungus, then sucking species should be resistant to this type of infection since they do not swallow solid food. The unsuccessful attempt to infect the *Dorylaimus* sp. with *H. anguillulae* supports this assumption. A further conclusion to be





drawn from this experiment concerns the use of fungi for the biological control of eelworms in soil. Most economically important species of eelworm are found among the sucking nematodes and obviously no successful control may be expected from the use for this purpose of *H. anguillulae* or of other fungi with a similar mechanism of infection.

This work is dedicated to Professor Paul Buchner, Porto d'Ischia (Napoli) on his seventieth birthday.

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EXPLANATION OF PLATES

PLATE 1

Different stages in the life history of Harposporium anguillulae

- Fig. 1. External hyphae emanating from the body of an eelworm. ($\times 250$).
 Fig. 2. The thick-walled cells of the endozoic mycelium in an infected eelworm. $\times 500$.
 Figs. 3-5. Different stages in the germination of the conidiospores on yeast-extract agar. Note the thin neck-like connexion between the spore and the initial hypha. $\times 450$.
 Figs. 6-8. Head parts of eelworms with germ tubes of the fungus originating in the oesophagus. $\times 250$.
 Fig. 9. Head region of an eelworm during an early stage of infection. The initial hypha grows inside the muscular layer of the oesophagus. $\times 500$.
 Fig. 10. An infected eelworm 1 month after death. The endozoic mycelium has been transformed into rounded chlamydospores. The external hyphae are already disintegrated, but their 'sockets', places of their former emergence from the cuticle, are still visible. $\times 500$.

PLATE 2

- Fig. 11. An eelworm infected with a *Harposporium* sp. strain species A. $\times 150$.
 Fig. 12. The same in higher magnification; note the arcuate shape and pointed tips of the conidiospores. $\times 720$.
 Fig. 13. Germination of a conidiospore of species A in the oesophagus of an eelworm. The germination filament penetrates the lining as well as the muscular layer of the oesophagus and the initial hypha starts its development in the body cavity of the eelworm. $\times 720$.
 Fig. 14. Development of the endozoic mycelium in an eelworm infected with species B. No involvement of the oesophagus. $\times 720$.
 Figs. 15, 16. Eelworms infected with species B. Note the rod-shaped spores. $\times 720$.
 Fig. 17. Spores of species B germinating on nutrient medium. $\times 450$.
 Fig. 18. A single spore of species B germinating on nutrient medium. A long thread-like germination tube connects the spore with the initial hyphae. $\times 450$.
 Figs. 19, 20. Conidiospores of species A germinating on a nutrient medium. The germination filament is visible. $\times 720$.

(Received 28 February 1958)

Since this article was written we have seen Drechsler, C. (*Bull. Torrey bot. Cl.* 1946, **73**, 557) describing *H. bysmatosporum* a species either identical with or very similar to our species B.

Influence of Corn Steep Liquor on the Oxidation of Ammonia to Nitrite by *Nitrosomonas europaea*

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SUMMARY: The oxidation of ammonia by *Nitrosomonas europaea* is accelerated in mineral liquid media by the addition of small amounts of corn steep liquor or its 'ash'. The amount of nitrite produced in such cultures usually exceeds the controls by 6-7 times after 6 days. However, in the following days nitrite-production becomes slower and after 14 days the controls have reached the same nitrite concentration as the corn steep liquor cultures. Concomitant with the accelerated nitrite-production hydrogen-ion concentration in the cultures increases considerably and may attain values as low as pH 6.3 after 14 days, as compared with c. 7.8 in the controls. The effect of corn steep liquor stimulation is more pronounced the smaller the inoculum used. Microscopical examination of slides immersed in cultures during incubation shows that *N. europaea* adheres to the glass surface only in cultures *without* corn steep liquor. The stimulatory factor in solutions of the 'ash' of corn steep liquor was not removed by dialysis nor by treatment with cation exchange resins. When the solutions were centrifuged both washed sediment and supernatant fluid were active. A possible explanation of the corn steep liquor effect is that colloidal matter in the solutions absorbs ammonia or any other nutrient in the medium in a way more favourable for their utilization by the organism.

The poor growth of autotrophic nitrifying bacteria in conventional simple chemically-defined media has been the subject of much speculation, and the opinion among many students of nitrification seems to be that one or more growth factors or inorganic micronutrients are needed for optimal growth of these organisms. This assumption is supported by two observations: (1) that nitrification usually proceeds faster in enrichment cultures than in pure cultures; (2) that viability of the bacteria in pure cultures tends to decrease. As it would be of great value for the study of the biochemistry of nitrification to obtain larger crops of organisms many attempts have been made to find growth-promoting substances. Unfortunately few of these attempts have met with success. However, Fred & Davenport (1921) reported that Nährstoff-Heyden (a preparation of egg albumin) stimulated nitrate formation by *Nitrobacter* spp.; this was later confirmed by Kingma Boltjes (1935) who found that the material was also effective for *Nitrosomonas* spp. Addition of 0.5% (w/v) Nährstoff-Heyden to a mineral agar medium resulted in larger colonies, and the same compound added to liquid mineral media inoculated with slowly nitrifying strains gave rise to enhanced ammonia- and nitrite-oxidation, respectively. Kingma Boltjes ascribed this effect of Nährstoff-Heyden to its ability to establish more favourable conditions of oxidation-reduction potential in the cultures. Winogradsky & Omeliansky (1899)

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reported that crude and ignited infusion of horse-dung, as well as soil extract, hastened the oxidation of ammonia in cultures. Hes (1937) also found that soil extract was active. Lees (1953) observed an irregular decrease in the lag-phase of *Nitrosomonas europaea* when biotin was added to the media. A stimulatory effect of iron has also been reported; Meiklejohn (1953) found that rather large quantities of ferrous sulphate (optimum at 6 mg. Fe/l.) stimulated oxidation of ammonia by the same organism in liquid mineral media.

The present communication comprises some results from experiments with corn steep liquor which has been found to accelerate the oxidation of ammonia by *Nitrosomonas europaea*. Unfortunately the organism degenerated and almost completely lost its ability to oxidize ammonia before this investigation was completed, and it has not yet been possible to obtain a new and active strain for the continuation of the experiments.

METHODS

The strain under investigation was isolated by Dr H. Lees and identified as *Nitrosomonas europaea*. The purity of the cultures was tested occasionally by transferring material to media able to support the growth of heterotrophic bacteria, viz. asparagine lucerne-extract agar, nutrient broth and a liquid mineral medium enriched with 0.5 % (w/v) glucose and 1 % (w/v) corn steep liquor. In cases where contaminants were found (rarely) the results were rejected.

A medium made up from tap water and the following salts was used throughout (% w/v): $(\text{NH}_4)_2\text{SO}_4$, 0.1; KH_2PO_4 , 0.025; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0125; NaCl, 0.0125; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0025; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.0025. Solid CaCO_3 (sterile) was added at c. 25 mg./5 ml. medium. The initial pH value was 8.0–8.3. This medium in 5 ml. lots in test tubes was autoclaved at 115° for 20 min. In most experiments the cultures were in triplicate, in some cases in quadruplicate. The incubation temperature was 25–28°.

Two different samples of corn steep liquor (CSL) were used. CSL stock solutions containing 1 % (w/v) of the material were prepared in distilled water, neutralized with N-NaOH, filtered, and sterilized by filtration through Seitz bacterial filters. CSL was added aseptically to autoclaved media. Solutions of ignited CSL (designated CSL-ash) were prepared as follows: water and volatile material of 20 g. crude CSL were evaporated in an oven at 105° for 2 days, and the hardened mass ignited at 600° in a quartz crucible. After cooling, 20 ml. distilled water were added and the suspension was boiled carefully for a few minutes, filtered through double filter-papers and the residue washed on the filter with 380 ml. hot distilled water. The filtrate appeared strongly opalescent and was used as stock solution without further clearance. This 5 % (i.e. equiv. 5 % CSL) solution was stored in the cold and, after dilution, added to the basal medium before autoclaving. Concentrations of CSL and CSL-ash referred to in the following paragraphs are expressed on an equivalent basis, namely, as % (w/v) of corn steep liquor.

Nitrate was estimated colorimetrically in a Coleman spectrophotometer, with the Griess-Ilosvay reagent.

RESULTS

Stimulation of nitrite formation by addition of CSL

In several experiments it was found that concentrations between 0.01 and 0.05 % of CSL resulted in a more rapid accumulation of nitrite in liquid mineral media inoculated with *Nitrosomonas europaea* (Table 1). Concentrations lower than 0.01 % had no effect, while 0.1 % and higher concentrations inhibited nitrification completely. Autoclaving a 0.02 % solution of CSL at 120° for 20 min. rendered it inhibitory, but autoclaving at 110° for 15 min.

Table 1. *Effect of corn steep liquor (CSL) on the oxidation of ammonia to nitrite by Nitrosomonas europaea*

Cultures sampled after incubation for 8 days at 25°.
Initial concentration in medium

CSL (%, w/v)	(NH ₄) ₂ SO ₄ (%, w/v)	NO ₂ -N formed (µg./ml.)
0	0	0
0	0.1	7.7
0.02	0	0.8
0.02	0.1	21.1

did not appreciably depress its stimulatory effect below that of a solution sterilized by filtration. Besides harmful organic substances in CSL it was thus evident that the material contained some factor which was able to stimulate either growth or respiration by *N. europaea* and the rather low effective concentrations made it seem probable that some organic growth substance or a mineral micronutrient might be responsible. In order to test the latter possibility a sample of crude CSL was ignited as described above and various concentrations of the resulting ash solution added to liquid media.

Stimulation of nitrite formation by CSL-ash

It was found that concentrations of CSL-ash corresponding to 0.01–1 % of the original CSL exhibited exactly the same stimulation of ammonia oxidation as did CSL itself. Maximum stimulation was found with 0.1 %; 0.001 % was almost without effect and 10 % was inhibitory. No additive effect was observed when CSL-ash (1 %) and CSL (0.02 %) were added simultaneously to the media. These results strongly pointed towards an inorganic stimulant.

The result of one of several identical experiments with 0.1 % CSL-ash is shown in Fig. 1. The stimulation (as measured by nitrite production) results in a decrease in the duration of the lag-phase and is most pronounced at the 6th day of incubation with an excess of nitrite-N in the CSL-ash cultures 6–7 times that of the controls. At the same time pH value in the cultures falls drastically, and when a pH value of *c.* 6.5 is reached nitrification becomes slower; after 14 days nitrite in the controls reaches the same level as in the CSL-ash cultures.

Role of size of inoculum

It was observed at an early stage that the size of inoculum greatly determined the results of the experiments. A fairly large inoculum (one drop, *c.* 0.05 ml.) containing suspended CaCO_3 from the stock culture gave irregular results and tended to hide the stimulatory effect of CSL. The best results were obtained with uniform droplets of the order of 0.01 ml., containing as little suspended matter as possible. It also proved to be important to use material

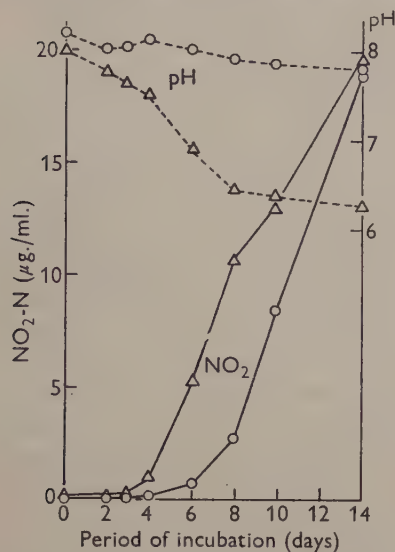


Fig. 1

Fig. 1. Nitrite production and pH value in cultures of *Nitrosomonas europaea*. Δ, 0.1% CSL-ash added; O, no addition of CSL-ash.

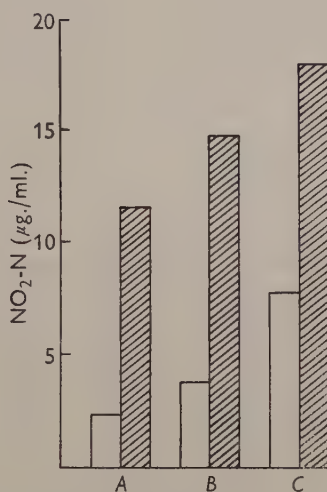


Fig. 2

Fig. 2. Effect of different sizes of inoculum on the nitrite production by *Nitrosomonas europaea*. Inoculum: A, one drop (*c.* 0.01 ml.); B, two drops (*c.* 0.02 ml.); C, five drops (*c.* 0.05 ml.). Hatched columns = 0.02% (w/v) CSL added; white columns = no addition of CSL.

from young (4–7 days) cultures as inoculum. The effect of different sizes of inoculum is shown in Fig. 2. With extremely small inocula the effect of CSL-ash was most pronounced (Fig. 3). In this experiment probably only a few organisms were carried over in the inoculum, resulting in the production of not more than 3 $\mu\text{g. NO}_2\text{-N/ml.}$ in the controls after 21 days, whereas in the CSL-ash media this amount was produced after only 5 days of incubation.

The role of the stimulating factor

After having established with certainty that crude as well as ignited CSL exhibited a favourable effect on the oxidation of ammonia by *Nitrosomonas europaea* it was of interest to find out whether the active material acted directly on the respiration process or indirectly by increasing the growth rate of the organism. A series of experiments dealt with in the following three paragraphs therefore were carried out.

Effect of CSL in solid media. Solid media were prepared from the basal medium with addition of 0.7–1.5% (w/v) agar (Difco Bacto-Agar or Difco Special Agar Noble). In several experiments growth and nitrite production in these media usually were more or less retarded when CSL (0.02%) or CSL-ash (0.1%) was added. Only in a single case was a favourable effect of CSL-ash observed: after 11 days small colonies c. 0.5 mm. diam. had formed on the CSL-ash media, while on the control media colonies were visible only with a hand-lens. Also, the nitrite reaction was stronger in the CSL-ash media. As the cultures grew older the difference between the two series disappeared.

On silica-gel medium covered with a thin layer of CaCO_3 , solution of the carbonate started first in the controls and after 15 days 145 $\mu\text{g. NO}_2\text{-N/ml.}$ was formed as compared with 105 $\mu\text{g./ml.}$ in gels containing 0.1% CSL-ash.

Experiments with suspensions of organisms + CaCO_3 . Cell material was obtained by growing *Nitrosomonas europaea* in 500 ml. lots of medium in 2 l. Erlenmeyer flasks, with occasional shaking. When all the ammonia had disappeared the cultures were centrifuged and the sediment (mainly consisting of CaCO_3 with adhering organisms) was washed free from nitrite with tap water. Finally, the material was resuspended in diluted (1/20) basal medium (no ammonia) and 50 ml. lots distributed in 300 ml. Erlenmeyer flasks. At zero time 1% $(\text{NH}_4)_2\text{SO}_4$ was added and nitrite determined immediately and later at regular intervals. With these 'resting' suspensions no stimulation of nitrite formation was noted within 12 hr. with 0.04% CSL, whereas a small stimulation was found with 0.5% CSL-ash. The results of these experiments are shown in Fig. 4.

Certain uncompleted experiments

As mentioned above, the strain of *Nitrosomonas europaea* used in this work died before the investigation was completed. However, a few of the uncompleted experiments nevertheless are of some interest and may be described briefly.

Microscopical examination of cultures. It was observed that the part of the test-tube wall in contact with the medium always became more milky or whitish when there was no CSL in the medium than when CSL had been added. As this appearance might have been due to differences in the adhesion of the organism to the glass surface, carefully cleaned slides (13 × 76 mm.) were immersed in ordinary 5 ml. portions of medium, with and without 0.1% CSL-ash. These culture vessels were autoclaved and inoculated in the ordinary way and the slides allowed to remain undisturbed in the tubes during incubation. The slides showed the same characteristic difference which had been observed on the test-tube walls: slides from CSL-cultures were clearer than those from the controls which showed an increasing whitish coating. After drying and fixation the slides were stained with phenol aniline blue and examined microscopically. Besides regular oval organisms of *Nitrosomonas europaea* evenly distributed over the surface many dividing organisms were seen. In one experiment the counts of organisms were 0.8 ± 2.0 (20 fields) in CSL-ash cultures compared with 48.5 ± 7.2 in the controls after 7 days of

incubation. In another experiment the corresponding figures were 15.8 ± 3.3 (10 fields) with CSL-ash and 135.3 ± 47.6 without CSL-ash after 12 days. These results show that the CSL-ash to a certain degree prevented the adhesion of the organisms to the glass surfaces. It therefore was expected that the number of free-living organisms in the liquid medium would be greater in the CSL-

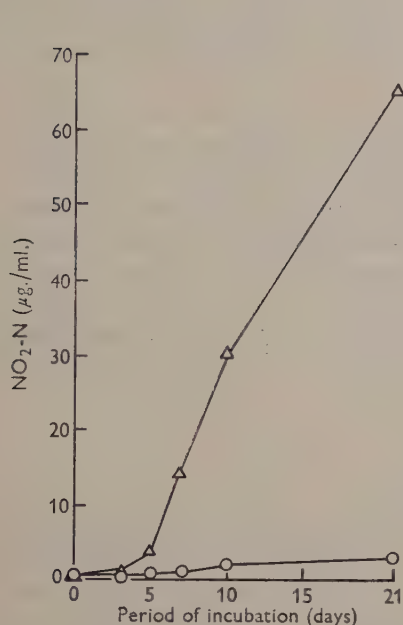


Fig. 3

Fig. 3. Effect of a very small inoculum on nitrite production by *Nitrosomonas europaea*. Δ = 0.1 % (w/v) CSL-ash added; \circ , no addition of CSL-ash.

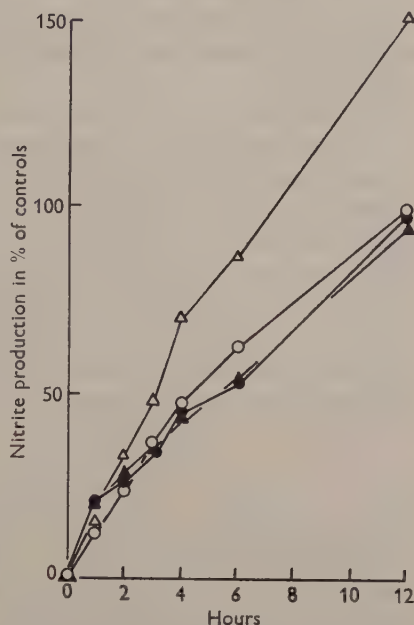


Fig 4

Fig. 4. Nitrite production in dense suspensions of *Nitrosomonas europaea*; (two separate experiments). Δ = 0.5 % (w/v) CSL-ash added; \circ , no addition of CSL-ash; \blacktriangle = 0.04 % (w/v) CSL added; \bullet = no addition of CSL.

ash cultures than in the controls; but this appeared not to be the case as judged by the examination of drops of culture medium: no macro- or microscopical difference could be observed with certainty.

Ion-exchange treatment of CSL-ash solutions. A 0.5 % (w/v) CSL-ash solution was run six times through an Amberlite IR-100 cation exchange column. Subsequently, the liquid was neutralized with *N*-NaOH, diluted and added to medium to give a final concentration of 0.1 % in the medium. This treatment did not remove the stimulatory factor from the CSL-ash. Samples of the treated CSL-ash solution as well as untreated CSL-ash solution were run through an Amberlite IR 4B anion-exchange column; the solutions thus treated inhibited nitrite formation. Since this was also true with distilled water, the inhibition was probably because of dissolution of small amounts of the phenolic resin.

Dialysis of CSL-ash solutions. CSL-ash solution was dialysed and the two

fractions thus obtained tested for stimulatory effect on nitrite formation. Ten ml. of 5% (w/v) CSL-ash stock solution (A) were dialysed against 90 ml. glass-distilled water (B) for 48 hr. at room temperature in a Visking bag 18/32. Fraction (B) was added to liquid media to give a concentration equivalent to 0.1% CSL-ash. The (A) fraction was further dialysed against two lots (1 l.) of glass-distilled water for a total of 96 hr. and finally diluted to give a concentration equivalent to 0.1% CSL-ash in the media. Only fractions (A) and (A)+(B) stimulated nitrite formation, whereas fraction (B) alone was without effect.

Centrifugation of CSL-ash solutions. CSL-ash solution (equiv. 5% w/v) was centrifuged and the effect of washed sediment (A) and supernatant (B) tested for stimulatory effect separately and in combination. Centrifugation for 1 hr. at 5000 rev./min. resulted in a water-clear supernatant fluid but both fractions separately and in combination gave the same degree of stimulation as did uncentrifuged CSL-ash solution. A new sample of solution was centrifuged for 90 min. at 13,500 rev./min. The higher speed and the longer centrifugation time somewhat decreased the stimulatory effect of the supernatant fluid (fraction B) indicating that the CSL-factor might be centrifuged down if a sufficiently high value of g were applied.

DISCUSSION

Corn steep liquor (CSL) has found manifold uses in microbiology mainly because of its high content in growth substances (Liggett & Koffler, 1948). The observation that small amounts of this material accelerated the oxidation of ammonia by *Nitrosomonas europaea* therefore made it seem probable that one or more organic growth substances were responsible for the effect. However, this assumption was disproved by the subsequent experiments with ignited corn steep liquor solids. As yet no one has demonstrated that organic substances are necessary, or will stimulate the growth or respiration by *N. europaea*. The effect of Nährstoff-Heyden has been ascribed to its stabilizing effect on the oxidation-reduction potential, and the observation that extracts of horse dung and soil can act as stimulating agents does not necessarily mean that organic substances in the extracts are responsible.

Besides organic growth substances corn steep liquor contains a variety of inorganic materials. By spectroscopic analysis Koffler, Knight & Frazier (1947) found some 20 known biological trace-elements in this material. It seems that it must be the mineral fraction of corn steep liquor which contains the stimulatory factor examined in this present work, but it seems unlikely that cations or undissociated small molecules are responsible for the effect, judging from the experiments with dialysed and centrifuged CSL-ash solution and CSL-ash solution treated in cation-exchange columns. Physically a CSL-ash 'solution' is a rather complex liquid consisting of various ions and salts in true solution, macromolecules in colloidal solution and finally small particles in suspension. Although nothing could be stated with certainty most results point towards the colloidal fraction as being the active agent in CSL-ash.

Possibly macromolecules act as absorbers of ammonia or any other nutrient in the medium in a way favourable for the utilization of these nutrients by the bacteria, or they might change the conditions in other ways when present in cultures of *Nitrosomonas europaea*. In this connexion it should be added that the author has also found a small stimulation with solutions of ignited Nährstoff-Heyden.

The inorganic colloidal matter present in CSL-ash solutions is most probably not present as such in corn steep liquor itself. However, as the latter is a product of corn (maize) seeds it will contain organic colloidal matter which might possibly act in a manner similar to the inorganic macromolecules.

Further support for the 'colloidal theory' might be deduced from experiments made by Meiklejohn (1953) who found that iron stimulated the oxidation of ammonia by *Nitrosomonas europaea*: 6 mg. Fe/l. given as ferrous sulphate to inorganic media (before autoclaving) produced optimal effect. However, it does not seem likely that this stimulation was wholly due to iron since 6 mg./l. is far more than is needed as a nutrient for *N. europaea*. The stimulation by iron must partly be due to other circumstances; either the presence in the ferrous sulphate of a mineral contaminant which is active, or the ferrous sulphate acts unspecifically. It is known that ferrous ion in alkaline solution will soon become oxidized to ferric ion, which in turn will react with water to form colloidal ferric hydroxide. In experiments carried out by the author (unpublished) it was found that even ferric hydroxide (equiv. 50 mg. Fe/l.) when added to the inorganic medium stimulated nitrite formation to the same degree as adding 0.1% CSL-ash solution. On the other hand, no stimulation was found with aluminium hydroxide or silica gel.

It is a pleasure to thank Dr H. L. Jensen for his interest in this investigation and for his many good advices. My thanks are also due to Professor T. Y. Kingma Boltjes, especially for a gift of Nährstoff-Heyden, and to Norsk Hydro for a grant.

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The Isolation and Estimation of the Poly- β -hydroxybutyrate Inclusions of *Bacillus* Species

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SUMMARY: Treatment of the cells of various *Bacillus* spp. with an alkaline solution of sodium hypochlorite resulted in dissolution of the cells and liberation of the intracellular lipid inclusion bodies. Analyses of the isolated and purified inclusions of *Bacillus cereus* grown under a variety of cultural conditions showed them to contain about 89 % poly- β -hydroxybutyrate and 11 % ether-soluble lipid. Parallel estimations of the poly- β -hydroxybutyrate content of intact organisms by Lemoigne's chloroform extraction method showed that all of it was present in the lipid inclusions. These observations form the basis of a simple and rapid method of estimating the poly- β -hydroxybutyrate content of *Bacillus* spp. It consists essentially of digesting a washed bacterial suspension with a standard alkaline hypochlorite solution under standard conditions and measuring the residual turbidity.

The intracellular lipid inclusions which form such a prominent feature of the large-celled species of *Bacillus* have been little studied. Their avidity for oil-soluble dyes such as Sudan Black clearly indicates their content of 'fatty' substances, but little is known of the nature of these or other materials that may be present. A notable advance was made by Lemoigne, Delaporte & Croson (1954) who were able to correlate the occurrence of lipid inclusions in *Bacillus* spp. with the presence in the cells of appreciable amounts of a polymer of β -hydroxybutyric acid, previously isolated from *Bacillus* 'M' by Lemoigne (1927). On the basis of these findings the authors concluded that the polymer was a constituent of the inclusions. Their view received some support from the observations of Weibull (1953), but has yet to be directly confirmed. The purpose of this paper is to provide such confirmation, and at the same time to describe a rapid method of estimating the poly- β -hydroxybutyrate content of small samples of organisms. The method is based on the observation of Meyer (1901) that when organisms of *Bacillus* spp. are suspended in an alkaline solution of sodium hypochlorite, they are almost completely dissolved, only the lipid inclusions remaining intact.

METHODS

Organisms. The laboratory strain of *Bacillus cereus* (strain SC) used had the following properties: motile; Voges-Proskauer positive; catalase positive; rapid liquefaction of gelatin and coagulated serum; β -haemolytic; produced acid on glucose, sucrose, maltose, glycerol and salicin but not on lactose, dulcitol, mannitol, arabinose, rhamnose, xylose, raffinose or inulin. Abundant

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spores and lipid inclusions were usually produced. An asporogenous mutant (strain AC) was obtained by ultraviolet irradiation of the above strain of *B. cereus*. It was recognized on an agar plate by the fact that old colonies remained fully rough instead of becoming gradually smoother as spore formation occurred. Apart from being completely asporogenous in all the culture media used, the mutant differed from the original strain in being non-motile. For optimal growth both strains required several amino acids, notably valine and leucine, but no other growth factors. The strain of *Bacillus megaterium* used was obtained from the National Collection of Type Cultures, Colindale, London (NCTC 7581). *Aerobacter aerogenes* (strain A3) was described by Duguid & Wilkinson (1953).

Cultural methods. Unless otherwise stated, all cultures of *Bacillus cereus* strain AC were grown aerobically at 37° for 40 hr. on a solid medium which contained (g./100 ml. distilled water): Japanese agar fibre (thoroughly washed in distilled water), 2; glucose, 1; Casamino acids (Difco), 0.005; NH_4Cl , 0.15; Na_2HPO_4 , 0.87; NaH_2PO_4 , 0.13; KCl , 0.02; Na_2SO_4 , 0.01; MgCl_2 , 0.002; CaCl_2 , 0.0001; MnCl_2 , 0.00006; FeCl_3 , 0.00002. The desired concentrations of glucose, NH_4Cl , phosphates, and Casamino acids were attained by adding concentrated solutions of these components, separately sterilized by light autoclaving, to the molten salts + agar base, just before pouring plates. The initial pH value of the medium was about 7.3. *B. cereus* (strain SC) and *B. megaterium* were grown on nutrient agar for 24 hr. to allow sporulation to occur. Small quantities of organisms were obtained by incubating cultures in Petri dishes containing 50 ml. agar medium. The inoculum, which consisted of 0.3 ml. of a just visibly turbid suspension of organisms in 0.85% (w/v) NaCl , was spread evenly over the surface of the medium to give confluent uniform growth. Large samples of organisms were obtained by incubating cultures in sterile enamel trays (40 \times 30 \times 2 cm.). Each of these trays held c. 1.5 l. medium which was surface-inoculated with 10 ml. of a dense suspension. During incubation, these cultures were protected with loose-fitting aluminium lids and serious contamination was never encountered.

Staining methods. Lipid inclusions were stained with Sudan Black (Burdon, 1946), volutin by Albert's method as modified by Laybourne (1924), and spores by the malachite green method of Ashby (1938). Approximate estimates of cell contents of lipid and volutin inclusions were made by microscopic examination of appropriately stained smears. Several hundred organisms were examined in each case and the average number and size of the inclusions/organism recorded in terms of an arbitrary scale of '+' signs. In the case of the lipid inclusions the scale ranged from '+' for an average of a few small inclusions/organism through '+' '++' and '+++' for increasing number of larger inclusions to '++++' for cultures in which nearly all the organisms were packed with masses of inclusions.

Turbidity measurements. The turbidities of suspensions of organisms and of lipid inclusions were measured with a 'Spekker' photoelectric absorptiometer, using a neutral filter. Concentrated suspensions were diluted to a point lying on the linear part of the curve relating turbidity to concentration.

Dry-weight measurements. The dry weights of suspensions of washed organisms in distilled water were estimated by drying measured volumes to constant weight at 120°. Suspensions of lipid inclusions were dried to constant weight at 60° in an oven, a procedure which gave the same weight as that obtained by freeze-drying.

Chemical estimations. Measurements of total nitrogen were made by a micro-Kjeldahl procedure and of total phosphorus by the Fiske & SubbaRow (1925) method. The ash content of isolated inclusions was determined by incinerating samples to constant weight in a platinum crucible.

Preparation of the standard alkaline hypochlorite reagent. This reagent, which was used for isolation and quantitative estimation of the lipid inclusions, was prepared as follows. Fresh bleaching powder (200 g.) was thoroughly triturated with a little distilled water and the volume made up to 1 l. A litre of 30 % (w/v) Na_2CO_3 was added with stirring, the mixture then being left to stand for 2–3 hr. with shaking at intervals, and finally filtered through paper. The pH value of the greenish yellow filtrate was adjusted to 9.8 with concentrated HCl, a flocculent precipitate which appeared during the course of this adjustment being removed by filtration after warming the solution to 37°. The resultant clear liquid was stored in a stoppered bottle in the refrigerator, and was stable for several months.

RESULTS

Microscopical observations

The appearance under the phase-contrast microscope of organisms of *Bacillus cereus* strain AC grown on the defined medium is shown in Pl. 1, fig. 1. The identity of volutin and lipid inclusions as seen with the phase-contrast microscope was established by examining particular fields of dried smears mounted in water, the smears then being stained for volutin or lipid and the same fields re-examined. The lipid inclusions occurred predominantly at the centre of the cell and varied in size from large spherical bodies as wide as the cell, often glassy blue in colour, to small brown or black opaque bodies with well-defined outlines. Volutin granules occurred almost solely at the poles of the cells and were just visible as rather indistinct smudges. The separate identity of the two types of inclusion was strikingly demonstrated when smears were stained simultaneously for volutin and lipid. For this purpose the counter-stain of Burdon's method was omitted and the smear taken instead through Albert's procedure. The lipid inclusions appeared brown against the green-stained cytoplasm, while volutin was jet black. The identification of spores under the phase-contrast microscope presented little difficulty. They were in any case absent from *B. cereus* strain AC, but in preparations of spore-bearing strains could readily be distinguished from the larger lipid inclusions by their uniform size and oval shape. Moreover, lipid inclusions were never stained by the malachite green spore stain, and spores invariably failed to take up Sudan Black.

When inclusion-bearing organisms of *Bacillus cereus* strain AC were suspended in the alkaline hypochlorite reagent at 37°, the turbidity of the sus-

pension decreased rapidly to a low but stable value. Examination with the phase-contrast microscope of samples taken at intervals showed that this turbidimetric change was accompanied by the dissolution of the cells, the lipid and volutin inclusions being released into the medium apparently intact. The cytoplasm faded rapidly during the first few minutes of the digestion, the inclusions at the same time becoming more distinct. In a short while all that could be seen of the cells were the inclusions encased in the by now weakly visible cell membrane. At a later stage the inclusions were to be seen in violent Brownian agitation, their movements, however, being apparently restricted by the barely visible remnants of the cell wall (Pl. 1, fig. 2, is an electron micrograph at this stage of digestion). Finally, the cell wall was completely broken down and the inclusions liberated into the surrounding medium where they persisted indefinitely. At first they tended to adhere in discrete clumps, but these eventually dispersed and the preparation had the appearance shown in Pl. 1, figs. 3, 4. Similar experiments with spore-bearing cultures of *B. cereus* (strain SC) *B. megaterium* and other *Bacillus* spp. revealed that spores also were apparently completely dissolved.

A more direct demonstration of the lysis of spore-bearing and of spore-free organisms was provided by continuous microscopic observation of the process. For this purpose, the action of the standard reagent (pH 9.8) on organisms between slide and coverslip was rather slow, and observations were facilitated by the use of a more alkaline reagent. When a wet film of unstained organisms was made in a mixture of 2 parts of the standard reagent and one part of 10N-NaOH, the digestion was complete in about 15 min. at room temperature, and continuous observation of individual organisms showed the lysis to follow exactly the course described above. A particularly striking demonstration of the process was made by applying the highly alkaline reagent to organisms which had been stained by suspension in ethanolic Sudan Black. Throughout the digestion the inclusions remained stained, and their persistence and eventual release into the medium was unequivocal. It is of some interest that when these stained organisms were suspended in the standard reagent (pH 9.8), the inclusions were rapidly decolorized. This behaviour, which will be referred to again in connexion with the staining properties of the isolated inclusions, did not impair the effectiveness of the experiment; the digestion was necessarily somewhat prolonged, but it was quite possible to locate stained inclusions at the start of the experiment and follow their fate during the whole of the lytic period.

Continuous observation of several spore-bearing strains undergoing lysis revealed that spores were released into the medium at the same time as the other inclusions, but in a few minutes they became grossly swollen, lost their glassy blue refractile appearance, slowly faded away and dissolved completely.

Turbidimetric observations

In order to study the turbidimetric changes accompanying the lysis of *Bacillus cereus* strain AC by hypochlorite, the turbidities of saline suspensions were measured and the organisms centrifuged down and resuspended in equal

volumes of hypochlorite reagent maintained at constant temperature. Samples were then withdrawn at intervals and their turbidities measured. The influence of pH value on the course of the digestion was studied by using batches of the reagent adjusted to different pH values with concentrated HCl or NaOH, the temperature during digestion being maintained at 37°; the effects of temperature were assessed by using the standard reagent at pH 9.8. Representative results are shown in Figs. 1 and 2. In all cases the onset of lysis was rapid, but the digestion gradually slowed until a steady final value of the turbidity was reached. This steady degree of turbidity was attained more quickly at higher temperatures and at higher pH values, but its value was essentially independent of either of these variables, and in the examples shown had been reached by between 60 and 90 min. Similar results were obtained with a variety of other *Bacillus* spp., including cultures bearing abundant spores. The relationship

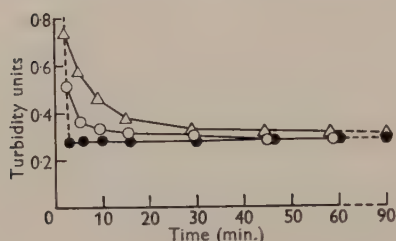


Fig. 1

Fig. 1. The effect of pH on the lysis of *Bacillus cereus* strain AC by standard hypochlorite at 37°. ●—●=at pH 11.6; ○—○=at pH 9.8; △—△=at pH 8.4. Initial turbidity=1.94 units.

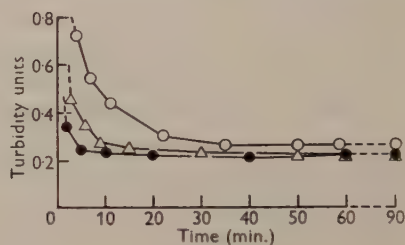


Fig. 2

Fig. 2. The effect of temperature on the lysis of *Bacillus cereus* strain AC by standard hypochlorite at pH 9.8. ●—●=at 48°; △—△=at 37°; ○—○=at 30°. Initial turbidity=2.40 units.

between the initial concentration of organisms and the value of the final steady turbidity attained on treatment with hypochlorite was studied by making a series of dilutions of a suspension of inclusion-bearing organisms of *B. cereus* strain AC and treating samples of these suspensions with hypochlorite (pH 9.8; 37°) in the manner described above, the final turbidity being measured after 90 min. digestion. It will be seen from the results (Fig. 3) that the final turbidity of a suspension containing not more than the equivalent of *c.* 10 mg. dry-wt. organism/ml. was directly proportional to the initial concentration of organism. The use of more concentrated suspensions led to a final turbidity that was disproportionately high, presumably because there was insufficient hypochlorite to complete the digestion.

The above results suggested that the final turbidity attained on treatment of the organisms was due to a hypochlorite-insoluble residue, and the nature of the cytological changes occurring during lysis left little doubt that this residue was comprised essentially of the lipid and volutin inclusions. It was, however, apparent from the appearance of the organisms under the phase-contrast microscope, that volutin granules were very much less refractile than the

lipid inclusions.¹ *Aerobacter aerogenes* grown under acid conditions which produce abundant volutin (Duguid, Smith & Wilkinson, 1954) gave a turbidity equivalent to that of *Bacillus cereus* grown under conditions giving a minimal lipid inclusion content. Moreover, observations made during extensive studies of the growth cycle of *B. cereus* strain AC under a variety of cultural conditions showed that this strain rarely formed much volutin. It was thought, therefore, that the volutin inclusions of this strain were unlikely to contribute greatly to the final turbidity attained on treatment with hypochlorite and that this turbidity could be used to provide an estimate of the cell content of lipid inclusions. To test this hypothesis, cultures of *B. cereus* strain

Table 1. *The relationship between the lipid inclusion content of cultures of Bacillus cereus Strain AC estimated microscopically, and the ratio of the turbidities before and after treatment with standard hypochlorite reagent.*

Microscopically estimated lipid inclusion content	\pm	+	++	+++	++++
Number of cultures studied	17	17	17	14	8
Mean ratio: $\frac{\text{final turbidity}}{\text{initial turbidity}}$	0.024	0.034	0.052	0.158	0.262
Standard deviation	0.006	0.006	0.018	0.024	0.011

AC containing widely different amounts of lipid inclusions (estimated by microscopical examination of stained smears) were prepared by incubation for varying periods on several versions of the basal medium, each of which had been made deficient in one of the main components, e.g. carbon, nitrogen, sulphur, potassium or phosphorus sources. Samples were treated with hypochlorite, the procedure used in this and all subsequent quantitative determinations being as follows. Washed organisms were suspended in 10 ml. 0.85% (w/v) NaCl to a density equivalent to c. 1.0 mg. dry-wt./ml., a concentration lying on the linear part of the curve which relates the final turbidity attained on treatment with hypochlorite to the initial concentration of organism (Fig. 3). The suspension was centrifuged, the supernatant liquid removed and replaced with an equal volume of standard hypochlorite reagent (pH 9.8) and the turbidity of the resulting suspension measured after 90 min. at 37°. The results are shown in Table 1, where the cultures have been grouped according to their microscopically-estimated lipid inclusion contents, and the average value for each group of the ratio of the turbidities calculated. It is clear that there was a direct statistical relationship between this ratio and the lipid inclusion content of the organisms. The scatter implicit in the standard deviations of these figures stems from the necessarily subjective nature of the microscopical estimation, rather than from inaccuracies inherent in the hypochlorite technique. This is shown by the results of the chemical analyses described below, which indicate that individual determinations with hypochlorite can be made to yield accurate estimates of the amount of lipid inclusion material.

In order to express this arbitrary measurement in more meaningful units, a

calibration curve was constructed relating the final turbidity of the hypochlorite-treated suspension to the mass of suspended lipid inclusions. Experience showed it necessary to ensure that the suspended inclusions used to prepare this curve were dispersed to the same extent as those of actual test samples, and that the colour and refractive index of the suspending fluid was as nearly as possible the same as that of the hypochlorite reagent at the end of the digestion period. The following procedure was therefore adopted. Washed inclusion-bearing organisms of *Bacillus cereus* strain AC were suspended to a density equivalent to c. 8.0 mg. dry-wt./l. in the standard hypochlorite reagent

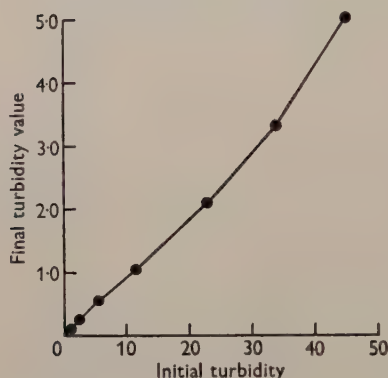


Fig. 3

Fig. 3. The relationship between the initial turbidity of a suspension of *Bacillus cereus* strain AC and the final turbidity after treatment with standard hypochlorite under standard conditions at pH 9.8 and 37°.

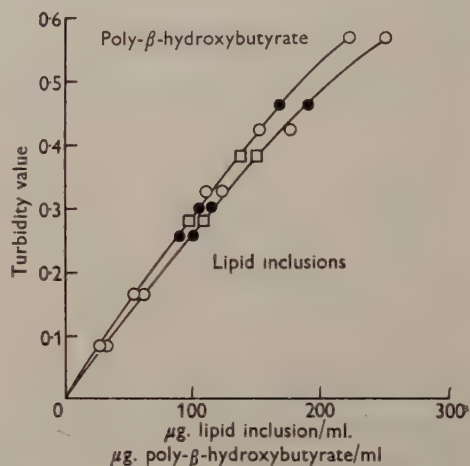


Fig. 4

Fig. 4. Calibration curve for the estimation of lipid inclusions and poly-β-hydroxybutyrate in *Bacillus cereus* strain AC.

at 37°. After stirring for 2 hr., the suspension was divided, one portion being centrifuged at high speed and the supernatant liquid passed through a fine sintered-glass filter to provide a quantity of 'used' hypochlorite. A series of dilutions of the other portion of the suspension were then made in this 'used' hypochlorite, and the turbidity of each dilution measured. Samples were centrifuged at high speed and the deposited inclusions resuspended as far as possible in distilled water and washed six times by centrifugation, a few drops of $\text{Na}_2\text{S}_2\text{O}_3$ being added to the water of the third wash in order to remove traces of free chlorine. Finally the dry weight of each sample was determined and the data plotted. Three cultures were used to prepare the calibration curve. They were grown on the standard medium for 30, 40, and 60 hr. and contained, respectively, 25.2, 16.0 and 9.7% of the dry weight as hypochlorite-isolated lipid inclusions. The calibration curve in Fig. 4 demonstrates that the method can be used for a wide range of inclusion content.

Chemical examination of the inclusions isolated by hypochlorite

A washed suspension of *Bacillus cereus* equivalent to *c.* 8.0 mg. dry-wt./ml. was centrifuged, suspended in the standard hypochlorite reagent at 37° and stirred for 2 hr. Attempts to wash the resulting dense suspension of inclusions by centrifugation were unsuccessful as the inclusions were prone to clump together at the bottom of the centrifuge tube, sometimes forming tough 'buttons' which were virtually impossible to resuspend. Consequently, the suspension was diluted with an equal volume of distilled water, and dialysed against distilled water for 24 hr. Volutin granules were found to be water soluble and were removed by this treatment. On adding sufficient NaOH to make the suspension slightly alkaline, the inclusions could be concentrated by low-speed centrifugation and resuspended fairly easily at this stage. Traces of free chlorine were then removed by the addition of $\text{Na}_2\text{S}_2\text{O}_3$ until a negative reaction was obtained with starch-iodide reagent. Finally the dialysis was repeated and the suspension freeze-dried to form a fine white powder containing 0.10 % N; 0.08 % P; 0.89 % ash. Exhaustive extraction by shaking with repeated changes of dry ether at room temperature removed about 11 % of the dry weight of the powder. Evaporation of the ether extract by a current of air at 60° yielded a brown greasy substance. Final traces of ether were removed from it by holding *in vacuo* over wax chips and it was dried *in vacuo* over P_2O_5 . The product did not show a sharp melting point; it contained 0.14 % N, 0.07 % P, had a saponification index of 218, an acid number of 60, and gave a positive acrolein test for glycerol (Feigl, 1947). These observations suggest a mixture of triglycerides, free fatty acids, and possibly traces of phospholipids.

The fraction remaining after ether extraction of the inclusions was found to be easily and completely soluble in chloroform and appeared to be a single compound. It had a sharp melting point (160°–169°), contained negligible traces of nitrogen and phosphorus, and had an ash content of 0.1 %. Evaporation of a chloroform solution yielded the material in the form of a thin greyish translucent membrane somewhat reminiscent of plastic sheeting. Its specific gravity, determined by flotation of small pieces in sucrose solutions of known density, lay between 1.23 and 1.25. It was soluble in a variety of solvents, including chloroform, glacial acetic acid, pyridine, octyl alcohol, 0.5 N-aqueous phenol, N-NaOH and triolein and was insoluble in water, ether, acetone, ethanol, carbon tetrachloride, the alkaline hypochlorite reagent and dilute mineral acids. On heating, the material melted, charred and gave off copious white fumes with an odour characteristic of crotonic acid. The fumes condensed on the cooler parts of the tube to form needle-shaped crystals of the latter acid, melting at *c.* 72°. The material was completely saponified by boiling for several hours with 2.5 N-NaOH, the saponification number being 640. All these properties suggested that it was a polymer of β -hydroxybutyric acid similar to that isolated from *Bacillus* 'M' by Lemoigne (1927), and described by him and by Képès & Péaud-Lenoël (1952). Confirmation of this finding was provided by an elementary analysis which gave an empirical formula of

($C_4H_6O_2$)_n. The molecular weight of the polymer, determined by isothermal distillation in chloroform, was about 5000, which suggests a chain length of about 60 residues.

Examination of samples of the inclusions isolated from many different cultures of *Bacillus cereus* strain AC showed that the proportion of polymer in the inclusions was essentially constant (c. 89%) and independent, over a wide range, of the inclusion content of the organisms (see, for instance, Table 2). It therefore seemed possible that the polymer contents of the organisms could be estimated fairly accurately as 89% of their lipid-inclusion content as

Table 2. *A comparison of the hypochlorite and chloroform-extraction methods of estimating the poly-β-hydroxybutyrate contents of cultures of Bacillus cereus strain AC*

Culture No.	'Lipid inclusion' content by hypochlorite method (% dry-wt. of organisms)	Poly-β-hydroxybutyrate content of isolated lipid inclusions (% dry-wt. of inclusions)	Polymer content of cells by hypochlorite method (% dry-wt. of organisms)*	Polymer content of organisms by chloroform extraction method (% dry-wt. of organisms)*
1	42.6	89.2	38.0	36.6
2	24.7	88.7	22.2	20.2
3	19.6	89.1	17.4	17.5
4	15.4	89.1	—	—
5	10.7	89.8	9.5	9.4
6	1.8	—	1.6	0.2

* Calculated from data in first column on assumption that the isolated lipid inclusions contain 89% poly-β-hydroxybutyrate.

indicated by the hypochlorite technique. The correctness of this assumption was shown by experiments in which the figures obtained by following this procedure with several cultures of *B. cereus* strain AC were compared with the polymer contents of the organisms as estimated by the chloroform extraction technique of Lemoigne & Roukhelman (1940). Large-scale cultures were used; one of them was grown on the defined medium containing 0.3% glucose, the rest were incubated for varying periods on the standard medium containing 1.0% glucose so as to yield organisms containing widely different amounts of lipid inclusions. Samples of these cultures containing the equivalent of c. 10 mg. dry-wt. organism were used for the hypochlorite estimations, the remainder (usually equiv. c. 5.0 g.,) being treated with chloroform. The results (Table 2) clearly show that the two results by the two methods agreed closely in the case of cultures containing between 9 and 40% of their dry weight as lipid inclusions. It thus became justifiable to calculate and construct the second curve shown in Fig. 4. which permits the poly-β-hydroxybutyrate content of a hypochlorite-treated suspension to be determined directly from the final turbidity. The discrepancy between the two methods in the case of cells containing only traces of the polymer is not understood. However, it does not detract seriously from the value of the hypochlorite method, the chief virtues of which lie in the small size of samples required and the rapidity with which accurate results can be obtained.

Staining properties of the isolated inclusions

The most significant result was obtained with the Sudan dyes. Both Sudan Black and Sudan III stained the inclusions *in situ*, the former an intense blue-black, the latter a pale red. Both these stains, however, failed to colour the isolated inclusions. This was not due to dissolution of a sudanophilic component in the unprotected inclusions by the solvent (70 %, v/v ethanol in water) used to carry the dyestuffs, since the isolated inclusions could not be stained even when mounted in saturated solutions of Sudan Black in either ethylene glycol or 50 % diacetin, both solvents which are almost incapable of dissolving lipids. Moreover, the lack of sudanophilia of the isolated inclusions was in agreement with the previously noted observation of the decolorization of Sudan Black-stained inclusions by the standard alkaline hypochlorite reagent. Thus it was clear that the isolated inclusions were no longer sudanophilic. No staining occurred with Albert's stain, Ashby's spore stain, the mercuric/bromphenol blue test for protein (Mazia, Brewer & Alfert, 1953) or the Feulgen reaction.

DISCUSSION

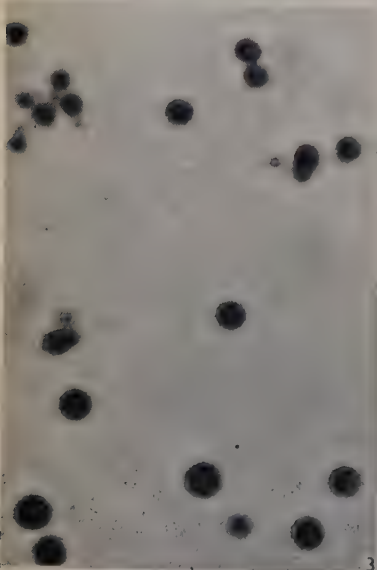
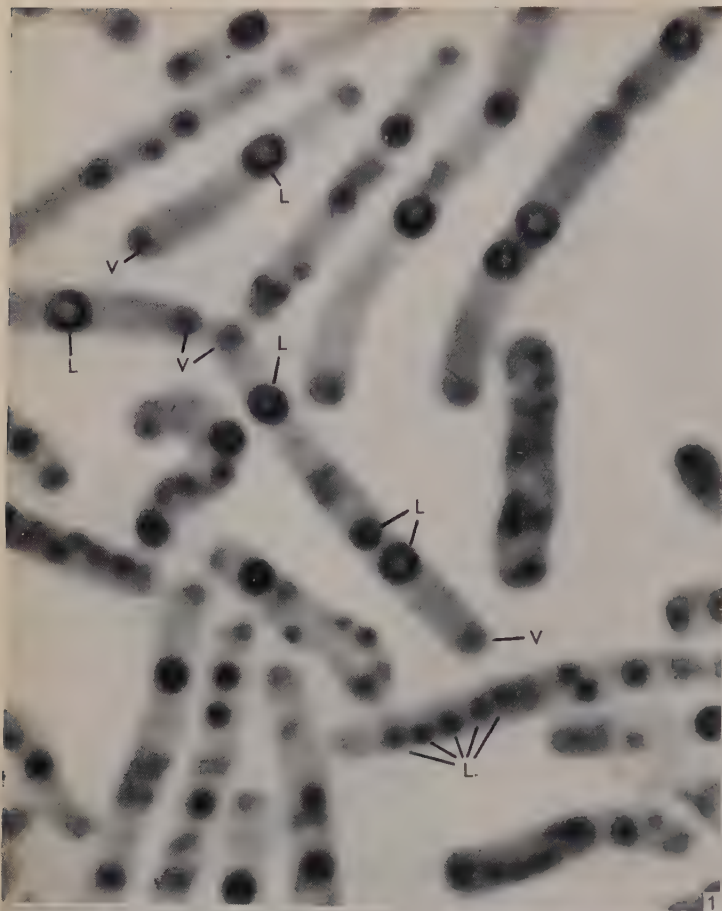
The hypochlorite method of estimating poly- β -hydroxybutyrate is rapid, simple to perform, and requires only the equivalent of *c* 10 mg. dry-wt. of organisms. It is thus particularly suitable for experiments involving frequent examination of a limited amount of culture material, and in the case of cultures containing moderate or large amounts of the polymer gives results which agree closely with those obtained by the much more cumbersome method of chloroform extraction. The discrepancy between the results given by the two methods in the case of organisms containing only traces of the polymer does not impose a serious limit on the practical utility of the method, since the 'large-celled' species of *Bacillus* usually contain considerable quantities of poly- β -hydroxybutyrate.

Although the asporogenous *Bacillus cereus* strain AC was the main subject of the present study there is no reason why the method should not be applicable to other members of the genus. A necessary preliminary to any such application is the construction of a calibration curve which relates the polymer content of cultures to the turbidities attained on treating the same cultures with hypochlorite. The calibration curve described in the present study was constructed by first determining the relationship between the turbidity and dry mass of the suspended inclusions, and then showing that the isolated inclusions contained approximately 89 % poly- β -hydroxybutyrate. Alternatively calibration can be achieved directly by relating the final turbidities attained on treating known concentrations of organisms with hypochlorite, to the amount of poly- β -hydroxybutyrate in the organisms, as determined by chloroform extraction. Thus Macrae & Wilkinson (1958) prepared a calibration curve for the polymer content of *Bacillus megaterium* strain KM. It should be noted, however, that the method cannot be validly applied to a particular strain unless it is first shown that the shape and position of the

calibration curve is independent of the cell content of polymer. This is most easily done by ensuring that several cultures containing widely divergent amounts of lipid inclusions are used for the preparation of the calibration curve. Some indication of the reproducibility of the method is given by the fact that, as the result of experience, duplicate determinations were considered unnecessary. A large number of tests made in duplicate showed that the difference between a pair of determinations was never more, and usually less, than 3% of their mean.

The poly- β -hydroxybutyrate content of the lipid inclusions of *Bacillus* spp. was first deduced by Lemoigne *et al.* (1944). Their evidence was indirect, being based on finding the polymer only in species bearing lipid inclusions. The present demonstration of the polymer in the isolated and apparently morphologically intact lipid inclusions of *B. cereus* provides a direct confirmation of their suggestion. Unfortunately, the method of isolation is such that little can be deduced about other possible constituents of the inclusions *in situ*. Any polysaccharides, proteins, etc., which might be present would be unlikely to avoid destruction or dissolution by the reagent unless they happened to be inaccessible to it, and the lack of these materials in the isolated inclusions does not rule out the possibility of their existence in the inclusions *in situ*. That some degradation of the inclusions does occur during isolation is shown by their loss of sudanophilia. It may be assumed that the inclusions *in situ* contain a sudanophilic component as an entity distinct from the poly- β -hydroxybutyrate. The existence of such a component is shown by the finding in this laboratory that small pieces of the polymer, extracted with either chloroform or hypochlorite, could not be stained with Sudan dyes, an observation that agrees with the statement of Cain (1950) concerning the non-sudanophilic nature of solid lipids. Thus the sudanophilia of the inclusions *in situ* is almost certainly not due to the presence in them of the polymer, but must be attributed to a sudanophilic component which is either removed by hypochlorite treatment or in some other way rendered non-sudanophilic. It is possible that the ether-soluble component of the isolated inclusions is an altered form of this sudanophilic material, but unfortunately it cannot even be assumed with certainty that the ether-soluble lipid is in fact a true constituent of the inclusions. It is conceivable that it merely represents cytoplasmic lipid which adheres to the inclusions during the isolation process. The answers to these problems are to be found in analysing lipid inclusions isolated by other and less drastic techniques.

The authors are grateful to Dr C. T. Greenwood for a molecular weight determination, to Dr J. W. Minnis for an elementary analysis, and to Dr J. P. Duguid for many helpful suggestions. One of us (D. H. W.) is indebted to the University of Edinburgh for the award of Post-graduate Studentships for the years 1953-55, and to the Agricultural Research Council for the award of a Research Studentship for 1955-56.



D. H. WILLIAMSON & J. F. WILKINSON—ESTIMATION OF POLY- β -HYDROXYBUTYRATE.
PLATE 1

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EXPLANATION OF PLATE

Cultures of *Bacillus* spp. grown for 40 hr. at 30° on the standard defined medium.

- Fig. 1. *Bacillus cereus* strain AC. Phase contrast of wet film. $\times 600$. Note lipid inclusions (L) and volutin inclusions (V).
- Fig. 2. *Bacillus megaterium* incompletely digested with hypochlorite. The lipid inclusions are still encased in a remnant of cell material. Electron micrograph of formalin-fixed organism, shadowed at 15° with gold + palladium. $\times 13,000$.
- Fig. 3. Lipid inclusions of *Bacillus cereus* strain AC, isolated after hypochlorite treatment. Phase contrast of wet film. $\times 5,000$.
- Fig. 4. Lipid inclusions of *Bacillus cereus* strain AC isolated after hypochlorite treatment. Electron micrograph after osmic acid fixation, shadowed at 15° with gold + palladium. $\times 15,000$.

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Poly- β -hydroxybutyrate Metabolism in Washed Suspensions of *Bacillus cereus* and *Bacillus megaterium*

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SUMMARY: Poly- β -hydroxybutyrate has been previously shown to be a major component of bacterial 'lipid' granules. In the present study, the conditions under which it was formed and degraded by *Bacillus cereus* and *B. megaterium* were studied in washed suspensions. Suitable substrates for synthesis were glucose, pyruvate or β -hydroxybutyrate. Acetate, although alone unable to induce synthesis, greatly enhanced formation in presence of these substrates. Under optimal conditions, suspensions synthesized up to eight times their original content of poly- β -hydroxybutyrate in 4 hr. Formation was inhibited by high concentrations of oxygen, although no synthesis occurred anaerobically in nitrogen. The optimal concentration of oxygen was about 5%. *B. cereus* only was able to synthesize poly- β -hydroxybutyrate in an atmosphere of hydrogen. In the absence of an external carbon and energy source, degradation occurred rapidly aerobically, to carbon dioxide and water, and more slowly anaerobically to β -hydroxybutyrate and acetoacetate. The evidence that poly- β -hydroxybutyrate is a reserve carbon and energy source is discussed.

Lemoigne (1923) showed that an aerobic spore-forming bacillus, designated *Bacillus* 'M' formed quantities of β -hydroxybutyric acid in anaerobic suspensions in the absence of an external carbon and energy source. In subsequent investigations (Lemoigne, 1925) he made quantitative estimations of this acid formed and concluded that it accounted for the greater part of the acidic substances produced under the conditions of his experiments. He then demonstrated (Lemoigne, 1927) that a substance having the empirical formula $(C_4H_6O_2)_n$ could be extracted from the bacilli by chloroform and he was able to show that the material was a polymer of β -hydroxybutyric acid. Subsequently it became clear (Lemoigne, Delaporte & Croson, 1944) that there was a correlation between the amount of this polymer which could be extracted and the amount of refractile 'fatty' cytoplasmic granular material exhibited by the bacilli. Confirmation that the polymer was a major constituent of these 'lipid' granules was obtained by Weibull (1953) in his observations on the nature of the granules isolated after dissolution of the cell wall of *Bacillus megaterium* by lysozyme. Williamson & Wilkinson (1958) showed that an alkaline hypochlorite solution would liberate the granules which upon recovery and analysis were shown to consist largely of poly- β -hydroxybutyrate. A rapid quantitative method for the estimation of this substance was also described. Lemoigne, Grelet & Croson (1950) drew attention to the different amounts of poly- β -hydroxybutyrate obtained by growing *B. megaterium* on different media, and Macrae & Wilkinson (1958) showed that more of this substance was formed as the glucose concentration of the growth medium was increased; the subsequent depletion of the product during the later stages of

growth suggested a storage function. Washed suspension experiments with non-proliferating organisms have hitherto been confined to the breakdown of poly- β -hydroxybutyrate. Tinelli (1955*a, b*) correlated this degradation with gas exchange observations.

The present work was undertaken to determine the conditions under which poly- β -hydroxybutyrate is formed or broken down and to gather information with a view to deciding upon its functional significance. *Bacillus cereus* and *B. megaterium* were used and the effects of various substrates, inhibitors and gaseous environments studied.

METHODS

Organisms and cultural methods. The asporogenous *Bacillus megaterium* strain KM (Northrop, 1951) and *B. cereus* strain AC (Williamson & Wilkinson, 1958) were chosen in order to avoid complication by spore formation. They were grown at 30° in a liquid medium which contained the following substances (g.) in 100 ml. distilled water: Na_2HPO_4 , 0.6; KH_2PO_4 , 0.3; NaCl , 0.3; NH_4Cl , 0.1; Na_2SO_4 , 0.01; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.001; Casamino acids (Difco), 0.01. Glucose was added to a final concentration of 0.3% or 2.0% (w/v) for the production of organisms respectively poor and rich in poly- β -hydroxybutyrate (Macrae & Wilkinson, 1958). The cultures were grown in glass tubes (50 \times 5 cm.) containing 500 ml. medium and oxygenated by passing filtered air from a small electric pump through a sintered-glass distributing tube. The bacterial inoculum was 5 ml. of an overnight culture obtained after training the organism to the medium by six previous subcultures.

Harvesting was effected towards the end of the log phase (about 18 hr.) and the organisms were washed twice in 0.85 (w/v) NaCl . All washed suspension experiments were carried out in flasks shaken in a water bath, the organisms being in 0.1 M- $\text{Na}_2\text{PO}_4/\text{KH}_2\text{PO}_4$ buffer (pH 7.2) except when stated otherwise.

Assay methods. Turbidity, dry weight, total nitrogen and poly- β -hydroxybutyrate estimations were carried out by the methods described by Williamson & Wilkinson (1958). The hypochlorite-isolated granules of *Bacillus megaterium* were found to contain a variable amount of ether-soluble lipid (between 5 and 10%); the calibration curve prepared and used for poly- β -hydroxybutyrate determination is given in Fig. 1. β -hydroxybutyrate and acetoacetate were estimated by the method of Thin & Robertson (1952).

Chromatography. Short-chain fatty acids were separated by the method of Duncan & Porteous (1953). Parallel experiments were made on the 2:4-dinitrophenylhydrazones of keto acids (Cavallini & Frontali, 1954).

Chemicals. Sodium 2-hydroxy-1-propane sulphonate was prepared by the method of Stewart & Cordts (1952).

RESULTS

The degradation of poly- β -hydroxybutyrate

Bacillus megaterium utilized its stored poly- β -hydroxybutyrate when a washed suspension of organisms made rich in poly- β -hydroxybutyrate by growth in the medium of high glucose content was shaken in presence of oxygen or anaerobically in presence of nitrogen. The results in Fig. 2 show that degradation was more rapid aerobically, causing a breakdown of 61 % poly- β -hydroxybutyrate compared with 17 % anaerobically. Comparable results were obtained with *B. cereus*.

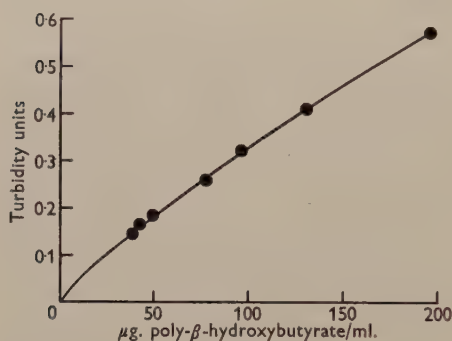


Fig. 1

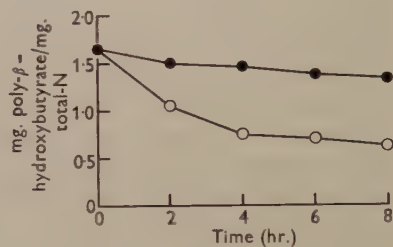


Fig. 2

Fig. 1. Calibration curve for the estimation of poly- β -hydroxybutyrate in *Bacillus megaterium*.

Fig. 2. Aerobic and anaerobic breakdown of poly- β -hydroxybutyrate in organisms. Flasks (150 ml.) contained 40 ml. washed suspension of *Bacillus megaterium* (equiv. 0.089 mg. total-N/ml.) in 0.1 M-phosphate buffer (pH 7.2). Gas phase air or nitrogen.

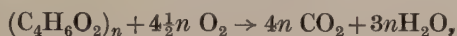
Chromatographic analyses of the supernatant liquids of centrifuged suspensions after anaerobic or aerobic breakdown showed the presence of β -hydroxybutyric acid, acetoacetic acid and smaller amounts of acetic acid, but no other aliphatic short-chain fatty acids. Amounts of β -hydroxybutyrate and acetoacetic acid after incubation in air, nitrogen or hydrogen for 200 min. are given in Table 1. The decrease in polymer of 160 μ g./ml. exhibited in presence

Table 1. *The production of β -hydroxybutyrate and acetoacetate correlated with the decrease of poly- β -hydroxybutyrate during the endogenous metabolism of Bacillus megaterium.*

Flasks (150 ml.) contained 10 ml. washed suspension *B. megaterium* (equiv. 0.59 mg. total-N/ml.) in 0.2 M-phosphate buffer (pH 7.2) Initial poly- β -hydroxybutyrate concentration = 2480 μ g./ml. Incubation time 200 min.

Gas phase	Decrease in poly- β -hydroxybutyrate (μ g./ml.)	β -Hydroxybutyrate formation (μ g./ml.)	Acetoacetate formation (μ g./ml.)
Air	580	8	45
Nitrogen	160	112	59
Hydrogen	160	116	51

of nitrogen or hydrogen represents 194 $\mu\text{g.}$ β -hydroxybutyric acid/ml. if a simple hydrolysis occurs. If acetoacetate were derived from β -hydroxybutyrate by oxidation as shown by Lemoigne, Péaud-Lenoël & Croson (1949) in washed suspensions of *Bacillus megaterium*, the overall yield of these acids from poly- β -hydroxybutyrate in presence of nitrogen or hydrogen was 89 and 87 %, respectively. These figures will be too high if other cellular materials are broken down to acetoacetic or β -hydroxybutyric acids. The remainder of the polymer may be further degraded to acetic acid and other products. There was no evidence for the presence of acetone formed during anaerobic breakdown, or of any gaseous products as determined manometrically. Aerobically, insignificant quantities of β -hydroxybutyrate were produced, although a much greater amount of poly- β -hydroxybutyrate was broken down and was probably oxidized to CO_2 and water. This contention is supported by parallel measurements of oxygen uptake and poly- β -hydroxybutyrate degradation. It was found, for example, that in an 8 hr. experiment where the poly- β -hydroxybutyrate concentration fell by 49 $\mu\text{g./ml.}$ (80–31 $\mu\text{g.}$) the suspension took up 130 $\mu\text{l. O}_2/\text{ml.}$ In accordance with the equation



only 57 $\mu\text{l. O}_2/\text{ml.}$ would be required for the complete oxidation of the amount of poly- β -hydroxybutyrate dissimilated.

The rate of endogenous respiration was probably dependent on the amount of poly- β -hydroxybutyrate in the organisms. Thus *Bacillus cereus* grown in media containing either 0.3 % or 2.0 % (w/v) glucose had poly- β -hydroxybutyrate/total-N ratios of, respectively, 0.83 and 3.27 and Q_{O_2} (N) values in the absence of an external substrate of, respectively, 169 and 536.

The effect of various substrates for the synthesis of poly- β -hydroxybutyrate

A washed suspension of *Bacillus megaterium* which had been grown on a medium of low glucose content to give a small initial quantity of intracellular poly- β -hydroxybutyrate, was shaken in air at 30° with a variety of compounds, all at 0.1M. The results (Fig. 3) show that, while there was a breakdown of poly- β -hydroxybutyrate in the control, synthesis occurred in presence of sodium pyruvate, sodium β -hydroxybutyrate and glucose; sodium acetate, although producing no net synthesis, prevented breakdown. It was further found that when sodium acetate was added to glucose, sodium pyruvate or sodium β -hydroxybutyrate, there was a considerably enhanced rate of synthesis so that the organisms more than doubled their content of poly- β -hydroxybutyrate during 4 hr. When different concentrations of sodium acetate (0.0025–0.3M) were added to a fixed glucose concentration (0.05M), using *Bacillus cereus* under the same conditions, the total amount of synthesis of poly- β -hydroxybutyrate was proportional to the acetate concentration up to about 0.05M and reached a peak at about 0.1M (Fig. 4).

The high values with glucose or pyruvate + acetate suggested that the former compounds might be producing an intermediate which reacted with acetate to give a 4-carbon compound related to β -hydroxybutyrate which then

condensed to give poly- β -hydroxybutyrate. Acetaldehyde was a possible intermediate and was tested by itself and in presence of acetate or pyruvate. The results (Table 2) showed no synthesis. In fact, acetaldehyde acted as a complete inhibitor of poly- β -hydroxybutyrate synthesis down to a concentration of 0.005M. A variety of other possible substrates were tested and are listed in Table 2. Insignificant synthesis of poly- β -hydroxybutyrate took

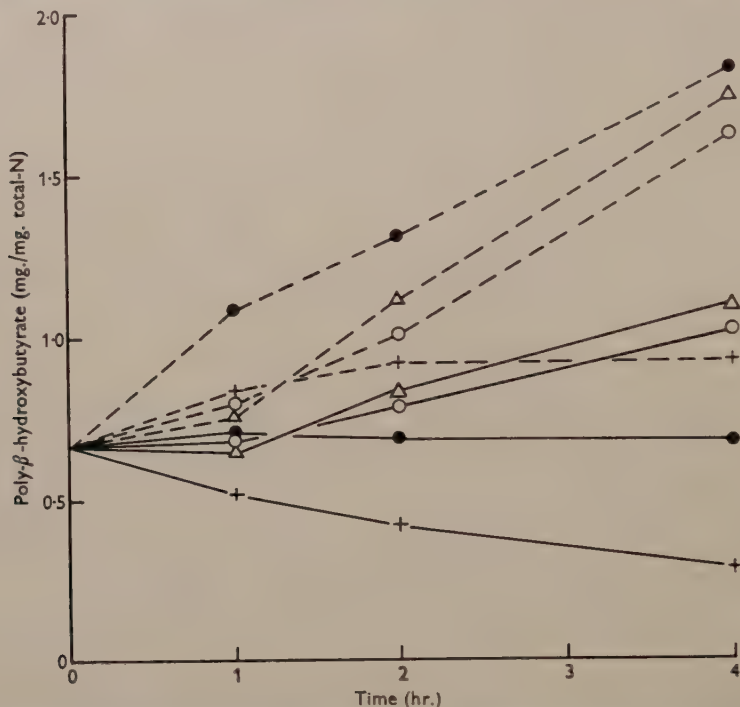


Fig. 3. The synthesis of poly- β -hydroxybutyrate by *Bacillus megaterium* from a variety of substrates. Flasks (150 ml.) contained 40 ml. washed suspension of *B. megaterium* (equiv. 0.133 mg. total-N/ml.) in 0.1 M-phosphate buffer (pH 7.2). Gas phase was air. +—+ = no substrate; ●—● = acetate; △—△ = pyruvate; ○—○ = β -hydroxybutyrate; +—+—+ = glucose; ○—○—●—● = β -hydroxybutyrate + acetate; △—△—●—● = pyruvate + acetate; ●—●—+—+ = glucose + acetate.

place with all the substances not previously tested. An analogue of β -hydroxybutyrate, sodium 2-hydroxy-1-propane sulphonate ($\text{CH}_3\text{.CHOH.CH}_2\text{.SO}_3\text{Na}$) was also inactive as a substrate. However, many substances, while not directly inducing synthesis of poly- β -hydroxybutyrate, partially suppressed its dissimilation. The permeability properties of the bacilli were not investigated and their inability to bring about synthesis of poly- β -hydroxybutyrate from many of the substances in Table 2 may have been due wholly or partly to their unavailability to the organisms.

It was possible that the increase in turbidity of the hypochlorite-isolated granules after incubation with these substrates in washed suspension might be due to the formation of volutin granules which Williamson & Wilkinson

Table 2. *The synthesis of poly- β -hydroxybutyrate by Bacillus megaterium from a variety of substrates*

Initial poly- β -hydroxybutyrate content = 31 $\mu\text{g./0.1 mg. total-N}$ of organism. Flasks (150 ml.) contained 40 ml. of washed suspension of *Bacillus megaterium* (equiv. 0.101 mg. total-N/ml.) in 0.1 M-phosphate buffer (pH 7.2).

Substrate	Conditions of incubation			
	2 hr.	4 hr.	+ Glucose (0.05 M)	
			2 hr.	4 hr.
			Poly- β -hydroxybutyrate content of organisms ($\mu\text{g./0.1 mg. total-N}$)	
None	26	24	36	35
Acetaldehyde	30	31	—	—
Acetaldehyde + acetate	30	31	—	—
Acetaldehyde + pyruvate	29	32	—	—
Acetate	32	30	62	87
Acetoacetate	32	31	36	33
Butyrate	28	28	36	37
Caproate	28	26	37	37
Citrate	30	22	37	36
Crotonate	28	28	36	40
Formate	28	26	37	36
Fumarate	29	29	38	36
Glycerol	32	28	34	33
2-Hydroxy-1-propane sulphonate	26	24	36	35
Propionate	31	28	33	33
Pyruvate	34	37	58	84
<i>n</i> -Valerate	31	30	36	36

(1958) showed were stable to the hypochlorite treatment. However, in experiments of a similar nature in which a microscopical estimation of volutin was made, there was no significant increase from the low initial value, whereas there was a considerable increase in the size of the sudanophilic 'lipid' granules. A second possibility was that the increased turbidity was due solely to an increase in the ether-soluble lipid rather than to poly- β -hydroxybutyrate, although the figures of Williamson & Wilkinson (1958) for the constancy of granule composition under a variety of growth conditions makes this unlikely. To investigate this point, parallel estimations of poly- β -hydroxybutyrate by turbidity and by chloroform extraction of the hypochlorite-isolated granules were carried out, before and after incubation with suitable substrates. A washed suspension (200 ml.) of *Bacillus megaterium* (equiv. 0.27 mg. total-N/ml.) in 0.1 M-phosphate buffer (pH 7.2) containing 0.1 M-sodium acetate and 0.05 M-glucose, was made up and an initial sample of 120 ml. taken for hypochlorite treatment under the standard conditions. The remainder was incubated aerobically at 30° for 4 hr. and then treated with hypochlorite. The turbidities were measured and the granules fractionated into ether-soluble and chloroform-soluble fractions by the method described by Williamson & Wilkinson (1958). The average result of two such experiments was as follows: poly- β -hydroxybutyrate calculated from the turbidity calibration curve, initial 102 $\mu\text{g./ml.}$, final 138 $\mu\text{g./ml.}$; chloroform-soluble material of isolated granules,

initial 98 $\mu\text{g./ml.}$, final 139 $\mu\text{g./ml.}$; ether-soluble material of isolated granules, initial 9 $\mu\text{g./ml.}$, final 15 $\mu\text{g./ml.}$ Thus the turbidity and chloroform-extraction methods for poly- β -hydroxybutyrate gave essentially the same result and the percentage of ether-soluble material in the granules remained virtually constant.

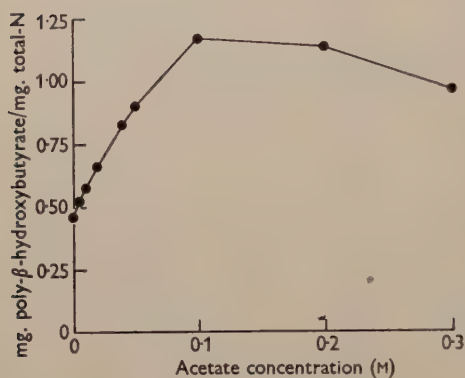


Fig. 4

Fig. 4. The synthesis of poly- β -hydroxybutyrate by *Bacillus cereus* from glucose (0.05M) in presence of different concentrations of sodium acetate. Flasks (150 ml.) contained 40 ml. washed suspension of *B. cereus* (equiv. 0.151 mg. total-N/ml.) in 0.1M-phosphate buffer (pH 7.2). Gas phase was air.

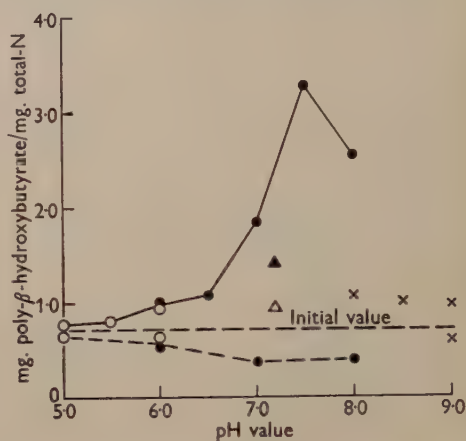


Fig. 5

Fig. 5. The effect of pH value and nature of buffer on poly- β -hydroxybutyrate synthesis by *Bacillus megaterium*. Flasks (150 ml.) contained 40 ml. washed suspension of *B. megaterium* (equiv. 0.043 mg. total-N/ml). Gas phase was air. --- = no substrate; — = 0.1M-sodium acetate + 0.05M-glucose; ○ = K phthalate + NaOH; ● = KH₂PO₄ + NaOH; × = H₃BO₃ in KCl + NaOH; △ = KH₂PO₄ + NaOH + 0.05M-tris + HCl; ▲ = KH₂PO₄ + NaOH + 0.005M-tris + HCl.

The effect of various environmental conditions on the synthesis of poly- β -hydroxybutyrate

In all the experiments on the effect of environmental conditions on the synthesis of poly- β -hydroxybutyrate from glucose and acetate, a washed suspension containing the equivalent of c. 0.1 mg. total-N/ml. was set up in 150 ml. flasks and shaken in air at 30°. The concentration of glucose was 0.05M and of sodium acetate 0.1M. The buffer was 0.1M-phosphate (pH 7.2) and the experimental period 4 hr., except where otherwise stated.

The effect of pH value was studied with *Bacillus megaterium* and a variety of buffers at 0.1M final concentrations. The results (Fig. 5) show that both synthesis and breakdown of poly- β -hydroxybutyrate have a fairly sharp optimum around pH 7.5. Both borate and tris (2-amino-2-hydroxy-methylpropane 1:3-diol) buffers, however, had an inhibitory effect, as shown by comparing the points in Fig. 5 with those of the corresponding pH value with phosphate buffer. This effect was not due to lack of phosphate since phthalate buffer (pH 6.0) gave essentially the same result as phosphate buffer (pH 6.0).

Further, when phosphate was added to tris or borate buffers, no increase in synthesis was noted. These effects of pH value on poly- β -hydroxybutyrate metabolism to some extent paralleled those of pH value on oxygen consumption which, with glucose as a substrate, gave an optimum at *c.* pH 7.0. However, although borate and tris buffers inhibited oxygen consumption, the effect was much less marked than on poly- β -hydroxybutyrate synthesis. Thus the addition of 0.05M-boric acid, 0.05M or 0.005M-tris buffer (pH 7.2) to 0.1M-phosphate buffer (pH 7.2) gave respectively 36, 31 and 8% inhibition of oxygen consumption on glucose.

The effect of adding a source of nitrogen or magnesium was next examined with *Bacillus megaterium*. Both gave a small inhibition of poly- β -hydroxybutyrate synthesis amounting to 15% with 0.001M-MgCl₂, 12% with 0.001M-NH₄Cl and 19% with 0.01M-NH₄Cl. With NH₄Cl, the inhibition was probably due to the intermediates and energy derived from glucose and acetate metabolism being diverted to processes connected with growth.

Sodium 2-hydroxy-1-propane sulphonate was tested as a possible competitive inhibitor of poly- β -hydroxybutyrate synthesis. Low concentrations of the analogue slightly stimulated synthesis (9% at 0.005M and 3% at 0.01M), while higher concentrations caused a slight inhibition (6% at 0.025M). These effects, although small, were reproducible and occurred at concentrations which had no effect on the rate of oxygen consumption under the same conditions. 2:4-Dinitrophenol, at 2.0×10^{-4} M, gave a 40% inhibition of synthesis although the rate of oxygen consumption was slightly stimulated (2.5%). This effect is in accord with the action of dinitrophenol as an inhibitor of carbon assimilation by preventing the synthesis of energy-rich phosphate bonds.

The effect of the gaseous environment on poly- β -hydroxybutyrate synthesis in *Bacillus cereus* was studied. Nitrogen, hydrogen, oxygen and air were used as the gas phases (frequently changed to keep the composition constant), the nitrogen and hydrogen being passed over heated copper to remove traces of oxygen. The results in Fig. 6 show the following points.

(1) Anaerobic conditions with nitrogen prevented synthesis. Observations with pyruvate (0.1M) and acetate (0.2M) also showed a complete inhibition of synthesis.

(2) Greater synthesis occurred in air than in oxygen, particularly over long incubation periods. In pure oxygen the organisms began to dissimilate their poly- β -hydroxybutyrate reserves during the later stages of incubation. The experiment was repeated with a variety of proportions of oxygen and nitrogen for an experimental period of 6 hr.; the results are given in Fig. 7. The optimal concentration of oxygen for synthesis was *c.* 5% (v/v). This sensitivity to oxygen was not annulled by adding reducing agents, such as cysteine. On the other hand, different substrates showed dissimilar degrees of sensitivity. Thus the percentage inhibition of poly- β -hydroxybutyrate synthesis in pure oxygen compared with air was measured with a variety of substrates (all at 0.1M concentration), using *Bacillus megaterium* over a 4 hr. experimental period. Glucose and acetate gave a value of 44%, pyruvate and acetate of 77%, and β -hydroxybutyrate and acetate of 11%.

(3) Hydrogen gave, surprisingly, almost as good synthesis as air. However, no evidence of the uptake of hydrogen could be obtained manometrically. In fact, there was a slight output of a gas which was not absorbed by NaOH and which might have been hydrogen (Q_{H_2} (N)=4). Potassium cyanide at 0.005 M gave a 71% inhibition of this synthesis, an effect possibly caused by action on hydrogenase which is said, under certain conditions, to be cyanide-sensitive (e.g. Hoberman & Rittenberg, 1943). The same concentration of cyanide gave a 94% inhibition of synthesis in air.

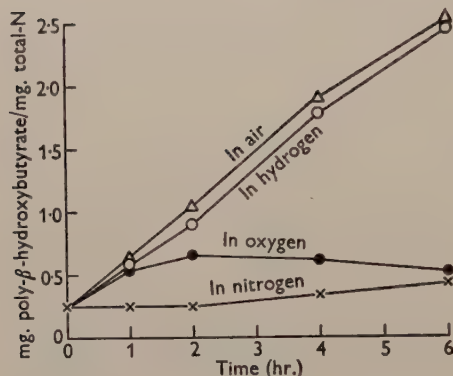


Fig. 6

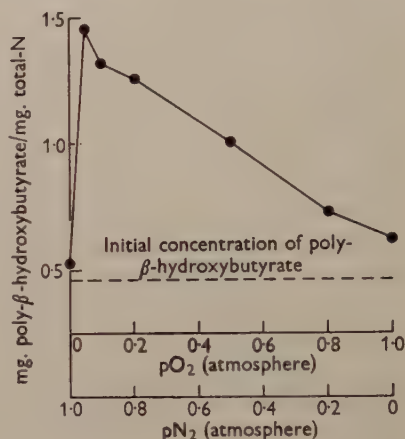


Fig. 7

Fig. 6. The effect of the gaseous environment on the synthesis by *Bacillus cereus* of poly- β -hydroxybutyrate from glucose (0.05 M) and sodium acetate (0.10 M). Flasks (150 ml.) contained 40 ml. washed suspension of *Bacillus cereus* (equiv. 0.150 mg. total-N/ml.) in 0.1 M-phosphate buffer (pH 7.2).

Fig. 7. The effect of various proportions of oxygen and nitrogen in the gas phase on the synthesis of poly- β -hydroxybutyrate from glucose (0.05 M) and sodium acetate (0.10 M) by *Bacillus cereus*. Flasks (150 ml.) contained 40 ml. washed suspension of *B. cereus* (equiv. 0.094 mg. total-N/ml.) in 0.1 M-phosphate buffer (pH 7.2).

(4) *Bacillus megaterium* gave essentially the same results as *B. cereus* (Figs. 6, 7), except that an atmosphere of hydrogen inhibited poly- β -hydroxybutyrate synthesis in much the same way as did nitrogen. This difference may be due to a difference in the amount of hydrogenase. However, these organisms also differ in their ability to form acid from glucose under anaerobic conditions. Measurements were made in bicarbonate, with 5% carbon dioxide in nitrogen as the gas phase and a glucose concentration of 0.1 M. In these conditions the equivalent of 5 mg. dry wt. *B. cereus* formed some 2.5 μ mole acid/hr. (corrected for endogenous acid production), whereas a similar mass of *B. megaterium* did not bring about any fermentation of glucose.

Another experiment was carried out to determine the effect of CO₂ on poly- β -hydroxybutyrate metabolism. No synthesis occurred with *Bacillus megaterium* when pure CO₂ was the gas phase and with 95% oxygen + 5% CO₂ (v/v) there was no stimulation of synthesis.

DISCUSSION

Various interpretations of the functional significance of bacterial 'lipid' granules have been put forward. Lemoigne (1927) described poly- β -hydroxybutyrate as a reserve material but, after further work (Lemoigne, Péaud-Lenoël & Croson, 1950) felt unable to decide whether it represented a waste or a storage product. However, Knaysi (1945) has adhered to the conception that the granules are lipoprotein particles arising from the cytoplasmic membrane which are not utilizable and may represent an abortive attempt at binary fission. Tinelli (1955*a, b*), on the other hand, found that a major part of the material was metabolized at sporulation and deduced that the two processes were intimately connected.

Poly- β -hydroxybutyrate is an ideal storage compound in so far as it is virtually insoluble in water and can thus be accumulated in large amounts as granules inside the cells. It is only weakly ionic and hence could also act as part of a neutralization mechanism by condensation of excess acidic products produced during metabolism to form this polyester. If such a neutralization mechanism were the primary function of poly- β -hydroxybutyrate, synthesis would probably have occurred best at a low pH value, but this has not been found in the present experiments. In the media used, the pH value never fell below 7.0 and yet the organisms formed up to 40% of their dry weight of poly- β -hydroxybutyrate; further, the optimum pH value for its synthesis in washed suspension was near neutrality. It is true that the external and internal pH optima may be markedly different, but it seems probable that the primary function of poly- β -hydroxybutyrate synthesis is not a neutralization mechanism. The possibility that poly- β -hydroxybutyrate is a storage compound is, however, further substantiated by the present work. In order to prove that a substance acts as a reserve carbon and/or energy source, it is necessary to demonstrate three main facts.

(1) It should best be formed in an environment containing an excess of an external carbon and energy source. Macrae & Wilkinson (1958) have demonstrated that organisms grown in a medium deficient in the carbon and energy source had a poly- β -hydroxybutyrate content many times lower (e.g. about four-fold) than organisms grown in a medium deficient in the nitrogen source only. In the present experiments, it was found that in washed suspension, a carbon and energy source was partially assimilated into poly- β -hydroxybutyrate, an assimilation which was prevented by 2:4-dinitrophenol. Under the best conditions, the proportion of poly- β -hydroxybutyrate rose from 3 to 20% of the bacterial dry-weight in 4 hr. It is to be expected that the main products of carbon assimilation will depend upon the nature of the carbon source. Thus Dagley & Johnson (1953) showed that when *Escherichia coli* was grown on media containing different amounts of glucose and acetate, lipid was formed best on media which contained an excess of acetate, while polysaccharide was formed best on media which contained an excess of glucose. Macrae & Wilkinson (1958) found that the presence of acetate in a glucose-containing medium yielded *Bacillus megaterium* in which the proportion of

poly- β -hydroxybutyrate was about 40% of the dry weight of organism as compared with a figure of 7% without the addition of acetate. The present experiments confirm the importance of acetate in the assimilation of carbon into poly- β -hydroxybutyrate.

(2) The supposed carbon and/or energy source should be capable of being broken down in the absence of an external carbon and energy source. Macrae & Wilkinson (1958), confirming the results of Lemoigne, Grelet, Croson & Le Treis (1945), showed that this occurred in the later stages of growth of *Bacillus megaterium*; the present experiments show that the process also occurs in washed suspension. Aerobically, dissimilation of poly- β -hydroxybutyrate in 4 hr. from 16 to 7% of the dry weight has been observed. Lemoigne (1925) showed that β -hydroxybutyrate was the main product of anaerobic breakdown in *Bacillus* 'M', and it was surprising to find acetoacetate as well as β -hydroxybutyrate produced anaerobically in the present work. The nature of the hydrogen acceptor which allows oxidation of the product of poly- β -hydroxybutyrate breakdown to acetoacetate is not known. No evidence of hydrogen formation was obtained manometrically. Under aerobic conditions carbon dioxide and water were the main products of endogenous breakdown, although acetoacetate was also formed in small amounts. Both the organisms used were asporogenous and these results are therefore at variance with the conclusions of Tinelli (1955*a, b*) who found that complete oxidation of poly- β -hydroxybutyrate only occurred in conjunction with sporulation, and deduced that the two processes were intimately connected. However, it may be concluded that this storage product together with polysaccharide will provide the carbon and energy required for sporulation in spore-forming organisms.

(3) The products of breakdown of the storage compound should be capable of use as a source of carbon and/or energy to prevent cell autolysis and death. If poly- β -hydroxybutyrate is broken down via β -hydroxybutyrate and acetoacetate or their derivatives as the anaerobic experiments suggest, and if acetoacetate can then be oxidatively metabolized through the coenzyme A compound of acetate to carbon dioxide and water, both carbon intermediates and energy would be produced. Certainly Lemoigne, Péaud-Lenoël & Croson (1950) showed that β -hydroxybutyrate could act as the sole source of carbon and energy for the growth of *B. megaterium*, although acetoacetate could be used only with additional carbon sources (malate, aspartate, glutamate). In confirmation of the ability of the organisms used here to utilize the products of poly- β -hydroxybutyrate breakdown, the present experiments show that the rate of endogenous respiration was higher in organisms rich in poly- β -hydroxybutyrate than in organisms poor in poly- β -hydroxybutyrate. Further, Macrae & Wilkinson (1958) showed that organisms rich in poly- β -hydroxybutyrate had a slower rate of autolysis than organisms poor in poly- β -hydroxybutyrate. It is probable, therefore, that poly- β -hydroxybutyrate acts as a reserve carbon and energy source.

The present observations also provide some clues to the pathway of synthesis of poly- β -hydroxybutyrate, although interpretation of such washed suspension experiments is hampered by the undoubted interference of perme-

ability factors. The stimulatory effect of acetate on synthesis from pyruvate, glucose or β -hydroxybutyrate might be explained in two ways. (a) Acetate combines with another two-carbon compound produced during the metabolism of the other substrates. This results in the production of β -hydroxybutyrate or a derivative of it (possibly via acetoacetate) which is then polymerized. Possibly the coenzyme A complex of β -hydroxybutyrate is the active compound. (b) Acetate, by means of a mass-action effect, alters the concentration of the intermediates in either the synthesis or the breakdown of poly- β -hydroxybutyrate so as to increase the rate of synthesis or to decrease the rate of breakdown. Isotope and cell-free extract experiments are under way in the hope of providing an answer to this problem.

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The Induced Synthesis of Hydrogenase by *Hydrogenomonas facilis*

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SUMMARY: *Hydrogenomonas facilis* lack hydrogenase activity when grown heterotrophically, but these organisms develop hydrogenase activity when incubated, in phosphate buffer, under an atmosphere of 95 % (v/v) hydrogen + 5 % (v/v) air. The development of hydrogenase activity is inhibited by 2:4-dinitrophenol and by chloramphenicol. The development is also inhibited by the addition of sodium lactate or sodium acetate unless a nitrogen source (e.g. ammonium sulphate) is present.

It has frequently been observed that hydrogen bacteria, grown autotrophically in an atmosphere of hydrogen + oxygen + carbon dioxide, show strong hydrogenase activity; yet the same bacteria, when grown heterotrophically, show little or no hydrogenase activity (Kluyver & Manten, 1942; Kistner, 1954; Kluyver & Verhoeven, 1954; Packer & Vishniac, 1955). Schatz & Bovell (1952) found that *Hydrogenomonas facilis* had some hydrogenase activity when grown in air on a variety of organic media, although the activity was much less than that of autotrophically grown bacteria. However, Wilson, Stout, Powelson & Koffler (1953) and Atkinson & McFadden (1954) were unable to demonstrate hydrogenase activity in *H. facilis* grown heterotrophically in air although Wilson *et al.* found that it was developed in organisms grown heterotrophically under diminished oxygen concentration (5 % O₂ + 5 % CO₂ + 90 % N₂). We also found that *H. facilis* had no hydrogenase activity when grown heterotrophically in air, but activity developed when these inactive organisms were incubated in a mixture of hydrogen and oxygen. Such behaviour suggests the induced synthesis of hydrogenase in the presence of hydrogen. The effect of some substances on the development of the hydrogenase activity is reported in this paper.

METHODS

The culture of *Hydrogenomonas facilis* was obtained from Dr A. Schatz. It was grown on the organic agar medium of Schatz & Bovell (1952) in air at 25° and harvested 45 hr. after inoculation. The organisms were washed and then suspended in sterile M/30 Sørensen phosphate buffer (pH 7.2) by passing a stream of either hydrogen or oxygen-free nitrogen.

A suspension of organisms (20 ml.) which never showed any hydrogenase activity initially, was incubated in sterile 250 ml. flasks which were filled with the appropriate gas mixture after evacuation. The flasks were shaken at 25° during the incubation period. In some experiments a continuous gas stream was passed through the cultures throughout the incubation period.

Hydrogenase activity was measured manometrically, on 2 ml. samples of suspension, by determining the hydrogen uptake from an atmosphere of pure hydrogen at 25°, with M/125 methylene blue as hydrogen acceptor. Hydrogen was purified before use by passage through 10% (w/v) KOH, 10% (w/v) CuCl_2 , 0.1 M- AgNO_3 and finally through water. All determinations were made in duplicate.

The dry weight of suspensions, both before and after incubation, was determined turbidimetrically by the aid of a calibration curve.

RESULTS

The induced formation of hydrogenase

Heterotrophically grown *Hydrogenomonas facilis* showed no hydrogenase activity when harvested but this activity developed when the organisms were incubated in an atmosphere of 95% (v/v) H_2 + 5% (v/v) air, although no appreciable increase of dry weight occurred (Fig. 1). A cell-free preparation having hydrogenase activity was obtained from the organisms after incubation, by breaking them in a Hughes press (Hughes, 1951). No such active preparation was obtained from the organisms before incubation in the hydrogen + air mixture.

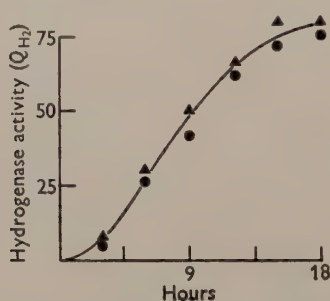


Fig. 1

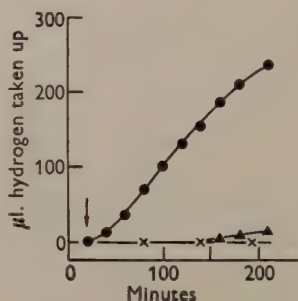


Fig. 2

Fig. 1. The time course of the development of hydrogenase activity on incubation in 95% (v/v) H_2 + 5% (v/v) air. Heterotrophically grown organisms were suspended in M/30 phosphate buffer (pH 7.2) and incubated during continuous passage of a stream of 95% (v/v) H_2 + 5% (v/v) air. Samples were removed at intervals and tested for hydrogenase activity. —▲—, Incubation flask 1; —●—, incubation flask 2.

Fig. 2. The effect of the composition of the gas phase during incubation on the development of hydrogenase activity. Heterotrophically grown organisms were suspended in M/30 Sørensen phosphate buffer (pH 7.2) and tested for hydrogenase activity after 24 hr. incubation in: (1) —●—, 95% (v/v) H_2 + 5% (v/v) air; (2) —x—x—, 100% (v/v) H_2 ; (3) —▲—▲—, 95% (v/v) N_2 + 5% (v/v) air. Equivalent dry weight of organism in each Warburg vessel = 0.65 mg.

Less hydrogenase activity developed when the organisms were incubated in 90% (v/v) H_2 + 10% (v/v) air and none at all when the gas phase lacked either hydrogen or oxygen (Fig. 2). The development of hydrogenase activity was inhibited when either 2:4-dinitrophenol (Fig. 3) or chloramphenicol (Fig. 4) was present in the incubation flasks; neither of these substances, at the concentrations used, had any effect on hydrogenase activity itself.

The development of hydrogenase activity is therefore dependent on the presence of both hydrogen and oxygen during the incubation period. Since the development of activity was inhibited by 2:4-dinitrophenol and by chloramphenicol (an inhibitor of protein synthesis; Wisseman, Smadel, Hahn & Hopps, 1954), it seems reasonable to conclude that some enzyme synthesis is necessary. Presumably the energy for the synthesis comes from the oxy-hydrogen reaction catalysed by the bacteria.

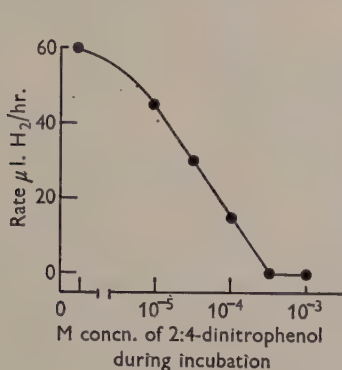


Fig. 3

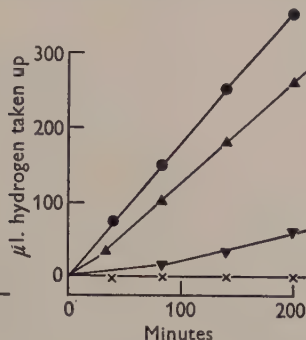


Fig. 4

Fig. 3. The effect of 2:4-dinitrophenol on the development of hydrogenase activity. Heterotrophically grown organisms suspended in $m/30$ Sørensen phosphate buffer (pH 7.2) were incubated for 24 hr. at 25° under an atmosphere of 95% (v/v) H_2 + 5% (v/v) air and in the presence of 2:4-dinitrophenol at different concentrations. The organisms were then assayed for hydrogenase activity. Equivalent dry weight of organism in each Warburg flask during assay = 0.85 mg.

Fig. 4. The effect of chloramphenicol on the development of hydrogenase activity. Heterotrophically grown organisms suspended in $m/30$ Sørensen phosphate buffer (pH 7.2) were incubated for 24 hr. at 25° under an atmosphere of 95% (v/v) H_2 + 5% (v/v) air in the presence of different concentrations of chloramphenicol. The organisms were then assayed for hydrogenase activity. Equivalent dry weight of organism in each Warburg flask during assay = 0.85 mg. Chloramphenicol concentration during incubation: —●—●—, 0 $\mu\text{g./ml.}$; —▲—▲—, 1 $\mu\text{g./ml.}$; —▼—▼—, 2 $\mu\text{g./ml.}$; —x—x—x—, 3, 5 or 10 $\mu\text{g./ml.}$

The effect of acetate and lactate on the development of hydrogenase activity

This was investigated in a number of experiments, and the effect of the addition of an inorganic source of nitrogen was also studied. In the earlier experiments, ammonium nitrate was added since *Hydrogenomonas facilis* grows autotrophically in a medium containing this (Schatz & Bovell, 1952). The results obtained were erratic, however, and the amount of growth (increase in turbidity) often poor. Analysis showed that nitrite accumulated in the medium when ammonium nitrate was present and further experiments showed that 10^{-3}M -nitrite inhibited hydrogenase activity. Later experiments were therefore carried out with 0.1% (w/v) ammonium sulphate as nitrogen source.

The organisms multiplied when incubated in phosphate buffer in the presence of sodium acetate or sodium lactate (0.1 or 0.2%), and growth was

much increased by the addition of ammonium sulphate. The addition of sodium acetate or lactate, however, stopped the development of hydrogenase activity unless ammonium sulphate was also present (Fig. 5). Clearly, hydrogenase formation is not always depressed by the addition of an organic

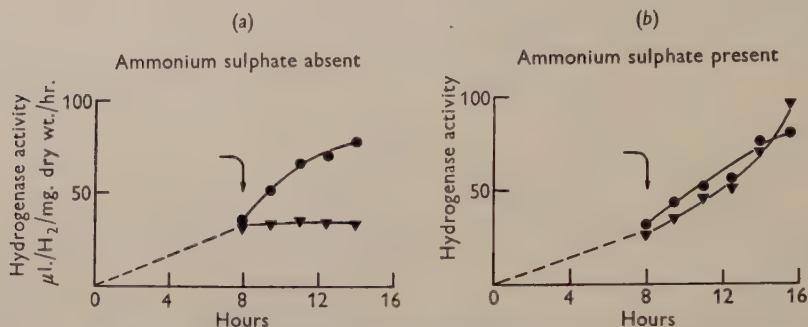


Fig. 5. The effect of lactate on the development of hydrogenase activity. Heterotrophically grown organisms suspended in $m/30$ Sørensen phosphate buffer (pH 7.2) were incubated under an atmosphere of 95 % (v/v) H₂ + 5 % (v/v) air. Sodium lactate (final concentration 0.2 % (w/v)) was added to one flask of each pair after 8 hr. incubation. Samples were withdrawn at intervals and assayed for hydrogenase activity. The incubation flasks were refilled with the H₂ + air mixture each time samples were removed. —●—, control; —▼—, lactate added. (a) Ammonium sulphate not added; (b) 0.1 % (w/v) ammonium sulphate present in both incubation flasks.

substrate. The situation resembles others in which the relative activity of an adaptive enzyme falls markedly under conditions of nitrogen deficiency (Virtanen & De Ley, 1948).

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The Influence of Growth Rate and Aeration on the Respiratory and Cytochrome System of a Fluorescent Pseudomonad Grown in Continuous Culture

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SUMMARY: A continuous culture apparatus was used to study the effects of air supply and of growth rate on the respiratory activities and the cytochrome content of a strictly aerobic fluorescent pseudomonad. The oxidizing capacity, measured as Q_{O_2} (succinate), of organisms grown with air as the growth-limiting factor was lower than that of organisms grown with succinate as growth-limiting factor, and with air supply in excess. There was no significant correlation between oxidizing capacity and growth rate under either of the two conditions. Estimation of the cytochrome content of whole organisms grown under these different conditions showed that this tended to decrease with increase in growth rate. Organisms grown with air as the growth-limiting factor always had about double the cytochrome content of cells grown at the same rates but with succinate as the growth-limiting factor.

The effects of aeration on the development and composition of microbial respiratory systems have been studied by a number of workers (Chin, 1950; Ephrussi & Slonimski, 1951; Chaix & Roncoli, 1950; Chaix & Petit, 1956, 1957; Hollman & Thofern, 1955; Lenhoff & Kaplan, 1953; Lenhoff, Nicholas & Kaplan, 1956). These studies have suggested that oxygen supply is one of the factors which govern the quantitative and qualitative composition of cytochrome systems in several facultative and obligate aerobic micro-organisms. In some of these systems oxygen appeared to act as an inducer of cytochrome formation (Chin, 1950; Ephrussi & Slonimski, 1951); in others, maximum cytochrome formation occurred only under conditions where the air supply was severely restricted (Lenhoff & Kaplan, 1953; Lenhoff *et al.* 1956). However, difficulties of interpretation arise since in most of the work cited the organisms were grown in batch culture, where cultural conditions could not be adequately controlled. Thus, in order to study the effects of oxygen supply on bacterial growth and metabolism, it is necessary to ensure that oxygen concentration is kept constant throughout the experiment. In batch culture, excess of oxygen or its complete absence may be easily ensured, but an exponentially increasing oxygen supply to keep pace with the increase in numbers of organisms is required to investigate the effects of concentrations between these limits. Otherwise, organisms at the beginning of the experiment are likely to be growing under different conditions of aerobiosis from those at the end of the experiment. Moreover, in batch culture, the rate of oxygen supply, if not in excess, will control the growth rate, at least for obligate

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aerobes. Differences obtained by varying the oxygen supply cannot, therefore, be analysed into direct effects due to changed oxygen concentration and indirect ones due to the altered growth rate.

By means of the continuous culture technique (Monod, 1950; Novick & Szilard, 1950; Herbert, Elsworth & Telling, 1956) these difficulties can be overcome. Once a steady state has been reached during growth in continuous culture, the organisms are growing exponentially in a constant concentration of nutrients (including oxygen) and metabolic products, and at predetermined rates. The effects of limiting concentrations of metabolites or air supply and of growth rate can, therefore, be investigated independently.

In the present work, the production of cytochromes and the oxidizing abilities of a strictly aerobic fluorescent pseudomonad were studied under conditions where the organism was growing exponentially, at different rates, and with either air supply or carbon supply limiting growth.

METHODS

Continuous culture apparatus. The apparatus employed was designed by one of us (R. F. R.) and will be described in detail in a later publication. It consists of a stirred and baffled growth vessel of approximately 300 ml. working capacity, which is fitted with a heater, thermostat and overflow. Stirring is provided by a magnet which is mounted in a nylon jacket and held in position by a shaft projecting from the centre of the lid. Air is passed to the bottom of the vessel and ejected beneath the stirrer, by means of a stainless steel syringe needle with a bent and flattened tip. Nutrient medium is supplied to the culture from a reservoir by means of a rubber tubing pump and Revco Zeromax speed changer (Sigmamotor Inc. Middleport, N.Y., U.S.A.). At the concentrations of organism and degrees of aeration used in these experiments, some foaming occurred in the growth vessel. To decrease this, small amounts (c. 0.1–0.3 ml.) of a 25 % (w/v) suspension of silicone Antifoam 'B' in distilled water, sterilized by autoclaving, were added to the culture as required.

The theory of this type of continuous culture apparatus has been extensively discussed by Monod (1950), Novick & Szilard (1950) and Herbert *et al.* (1956), who have derived the mathematical equations for the relationships between concentration and rate of supply of nutrients and the concentration and rate of growth of organisms in the culture. They have shown that in a steady state, the specific growth rate (μ) of the organisms is equal to the dilution rate (D = rate of medium flow/volume of culture). This results because the specific growth rate is a function of the concentration, in the culture, of the growth-limiting factor. The growth rate of the organism can, therefore, be controlled by the rate of flow of medium, whilst the concentration of organisms in the culture at any given growth rate is controlled by the initial concentration of the growth-limiting factor.

Organism. The organism used was a strictly aerobic fluorescent pseudomonad, strain KB1 (Kogut & Podoski, 1953) which produces a green fluorescent, water-soluble pigment and does not reduce nitrate. It was maintained on

10 % (w/v) yeast autolysate (Barker & Beck, 1942) agar slopes by monthly transfer. For inocula a loopful from the slope was transferred to 10 ml. of 10 % (w/v) yeast autolysate in a 100 ml. conical flask and grown for 6–8 hr. at 30° with shaking; 5–10 ml. of such a culture were used to inoculate 300 ml. of the continuous culture medium.

Medium. The composition of the medium was as follows: 1 % (w/v) sodium succinate, 6H₂O; 0.25 % (w/v) NH₄Cl; 0.125 % (w/v) Na₂HPO₄; 0.875 % (w/v) KH₂PO₄; the following trace elements (μ g./ml.): 61 Mg as MgO; 8 Ca as CaCO₃; 11.2 Fe as FeSO₄.7H₂O; 3.27 Zn as ZnSO₄.7H₂O; 2.75 Mn as MnSO₄.4H₂O; 0.64 Cu as CuSO₄.5H₂O; 0.59 Co as CoSO₄.7H₂O; 0.10 B as H₃BO₃. The medium was made up in 9 l. batches and sterilized by autoclaving at 15 lb./sq.in. The pH value of the sterilized medium was *c.* 6.0. When the organism grew and succinate was consumed, the pH value increased and the high buffer concentration kept it at pH 7.0–7.25 under conditions where succinate was the limiting factor, and at about pH 6.6–6.8 under conditions where oxygen was limiting.

Air supply. Air was supplied to the culture from a compressed air cylinder and was passed through a heated furnace and close-packed glass-wool filter to ensure sterility. The air flow into the vessel (in ml./min.) was measured by a Venturi meter. Since only these rates of air flow and not the amount of dissolved oxygen in the culture could be measured, we satisfied ourselves that carbon and not oxygen was the limiting factor under conditions of 'full aeration' by the following criteria. (a) In preliminary experiments we found that under similar conditions of aeration, but with a richer medium, this organism grew at a similar rate, but to *c.* 4 times the concentration of organisms here achieved. (b) Increase of air supply at constant rate of medium flow did not cause an increase in the concentration of organisms. (c) The concentration of organisms did not fall appreciably when the flow-rate of the medium was increased, indicating that air supply was not limiting the growth rate of the organisms. (d) The oxygen solution rate in the culture vessel, under the conditions of growth, was measured by the sulphite oxidation method of Cooper, Fernstrom & Miller (1944) and was found to be 504 mmole O₂/hr./l. As has been pointed out by Pirt (1957), this represents the maximum amount of available oxygen, and Wise (1951) has reported that measurement of oxygen solution rates by the sulphite method gives higher values than do polarographic measurements. However, the measured oxygen solution rate was at least 10 times in excess of the probable oxygen demand of the highest concentration of organisms in the culture, growing at the fastest rate which we used. This could be estimated, either from the maximum rate of succinate oxidation by washed suspensions, or from the growth rate and succinate concentration supplied in the medium, according to the equation given by Pirt (1957), assuming that the succinate is oxidized to completion with the uptake of 3.5 mole oxygen/mole of succinate. (If only 2.5 mole oxygen are consumed/mole succinate utilized, then the oxygen solution rate is 14 times in excess of the oxygen demand of the culture.)

Determination of amount of growth. For routine determinations of concen-

tration of organisms, the optical densities of samples were measured, after suitable dilution in distilled water, in a Hilger Spekker absorptiometer with neutral grey filter, against a water blank. The dry weight of the sample in mg./ml. was then calculated from a calibration graph, prepared from measurements on a sample of the organism growing in the logarithmic phase in batch culture. It has been suggested (Pirt, 1957) that the size (and hence the optical density for a given mass concentration) of an organism growing in continuous culture, varies somewhat with growth rate. We, therefore, also carried out 'direct' dry-weight determinations on samples growing under the various conditions. For this purpose, samples were centrifuged, after filtration through a coarse fluted filter-paper to remove precipitates, and the deposit

Table 1. *Concentrations of pseudomonad KB1 in continuous culture growing under different conditions*

Dilution rate (D (hr. ⁻¹))	(a) Concentration determined turbidimetrically (mg. dry wt. organism/ml.)	(b) Concentration determined gravimetrically (mg. dry wt. organism/ml.)	Difference (%)	Ratio a/b
Carbon-limited states				
0.142	1.28	1.36	5.9	0.94
0.142	1.09	1.44	24.3	0.75
0.228	1.39	1.48	6.0	0.94
0.228	1.35	1.45	6.8	0.93
0.396	1.41	1.47	4.1	0.96
Air-limited states				
0.072	0.70	0.68	2.9	1.03
0.072	0.70	0.79	11.4	0.89
0.144	0.53	0.56	5.4	0.95
0.144	1.03	0.97	6.2	1.06
0.216	0.61	0.59	3.4	1.03
0.216	0.46	0.46	0	1.00
0.378	0.71	0.68	4.4	1.04

washed 3 times with distilled water. The washed organisms were then dried at 105° to constant weight. Table 1 shows the values for concentrations of organisms determined turbidimetrically and gravimetrically on the same samples taken from the various steady states.

It can be seen that they do not differ by more than 7 % (except for two of the samples), and that the ratios of the dry weights obtained by the two methods do not appear to vary with growth rate. There does appear to be a difference in the average of the ratios (of the two dry-weight values) obtained from the carbon-limited and the air-limited steady states, but this was too small to affect the results of measurements (such as Q_{O_2} , cytochrome content, etc.) based on dry weights of samples determined turbidimetrically. For the two samples which gave differences for the two dry-weight determinations of 24 and 11 % respectively, duplicate determinations were carried out on further samples from the same steady states, and these agreed well with the rest of the values obtained.

Measurement of oxidizing ability as rate of succinate oxidation. The oxidizing abilities of organisms grown under the various conditions described were determined by measuring the rate of oxygen uptake by washed suspensions with succinate as substrate. For this purpose, samples of culture were collected, filtered through coarse fluted paper to remove any precipitates, centrifuged and the deposit washed once with distilled water. The organisms were then suspended in distilled water to concentrations equivalent to 2–3 mg. dry weight organism/ml. and the rate of oxygen uptake in $\mu\text{l./hr./mg. dry wt. of organism}$, in the presence of excess succinate ($=Q_{O_2}$ succinate) determined manometrically in the usual manner at 30° and pH 7.4. Duplicate determinations were made on each sample and the results averaged. The rate of endogenous respiration of each sample was also measured, but this was invariably very small compared to the rate of succinate oxidation (less than 5 %) and was ignored.

Chemical estimations

Samples for chemical estimations were immediately acidified by collecting in tubes containing 0.5 ml. 10N- H_2SO_4 .

Succinate was estimated by the method of Krebs (1937).

Steam-volatile acids were estimated by distillation in the apparatus of Markham (1942), and titration with standard NaOH in a stream of CO_2 -free air.

Keto-acids were estimated by a method for which we are indebted to Dr E. R. Stadtman (personal communication, 1957). A portion of an acidified sample was centrifuged and to a volume of the supernatant fluid, containing from 0.1 to 0.25 μmole keto-acids, was added 1 ml. of M-acetate buffer (pH 4.9) and 0.5 ml. of 0.05M-thiosemicarbazide, and the volume made up to 5.0 ml. with distilled water. The solutions were incubated at room temperature for 30 min. and the optical density at 290 $\text{m}\mu$ read in the Unicam SP 500 spectrophotometer against a reagent blank containing water instead of the sample. The amounts of keto-acids expressed as $\mu\text{mole pyruvate/ml.}$ were then determined from a calibration graph prepared with a standard solution of sodium pyruvate.

Estimation of cytochrome content of whole organisms

The absorption spectrum of whole organisms, when measured in a spectrophotometer in the conventional way, with a water blank in the second cuvette, is distorted by light scattering. Shibata, Benson & Calvin (1954) (see also Keilin & Hartree, 1955) found that the effect of scattering could be minimized by inserting a diffusion screen between the cuvette and the photocell of the spectrophotometer. They found that either opal glass or a strip of filter-paper soaked in medicinal paraffin proved satisfactory for the purpose.

Fig. 1 shows the absorption spectrum of a suspension of pseudomonad KB1 which had been treated with a few crystals of dithionite to ensure complete reduction of the cytochrome. This spectrum was obtained with a Quartz prism Hilger Uvispec spectrophotometer fitted with a diffusion screen

made from Whatman No. 3 filter-paper soaked in medicinal paraffin and mounted between two glass slides. The slit width was 0.5 mm. and the band width in the range 410–430 $m\mu$ was 3–4 $m\mu$ and in the range 540–600 $m\mu$ was 5–7 $m\mu$. This absorption spectrum shows α -, β - and γ -bands, similar to those of crude and purified extracts of pseudomonad KB1 (Kogut, 1957). The γ -band (which was unsymmetrical) had a maximum at 418 $m\mu$. The optical density (OD) at 418 $m\mu$ of a suspension equivalent to 5 mg. dry wt. organism/ml. was of a magnitude, such that it seemed possible to use this method to

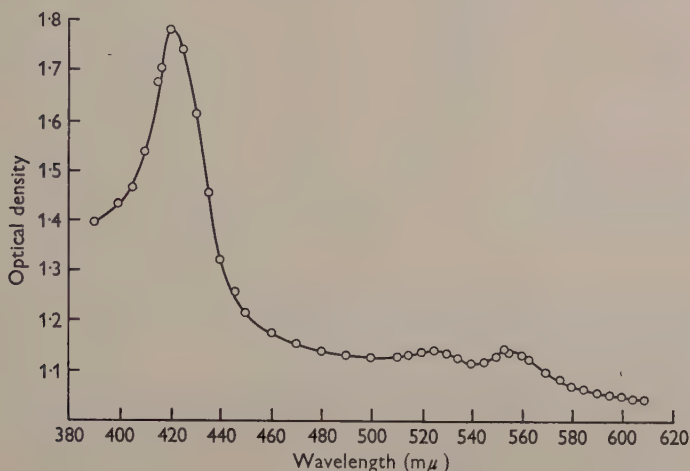


Fig. 1. Reduced absorption spectrum of a washed suspension of pseudomonad KB1. Suspension concentration equivalent to 5.6 mg. dry wt. organism/ml. Absorption measured with a quartz prism Hilger Uvispec Spectrophotometer fitted with a diffusion screen of Whatman No. 3 filter-paper soaked in medicinal paraffin. A few crystals of sodium dithionite were added to the suspension to ensure complete reduction.

obtain an estimate of the cytochrome content of whole organisms. The OD at 418 $m\mu$ is the sum of three components: (a) the absorption of the cytochrome pigments; (b) the absorption due to components other than cytochromes; (c) the scattering not compensated for by the diffuser. Both the non-specific absorption and scattering may be evaluated approximately by measuring the OD at 500 $m\mu$, a wavelength where the absorption due to cytochrome is least. The difference between the OD at 418 $m\mu$ and the OD at 500 $m\mu$ which we call Δ , will approximate to the OD of the cytochrome of the suspension.

Chance (see Chance & Williams, 1956) used a similar method for calculating the cytochrome content of particulate preparations. Figure 2 is a plot of Δ against mg. dry wt. organism/ml.; it will be seen that the curve is approximately linear over a limited range of concentrations of organism. By measuring OD (418) and OD (500) at a constant concentration of organisms, the correction factor is made more precise and the values of Δ so obtained are likely to represent more accurately the cytochrome content of the various suspensions. To test the procedure further, varying amounts of horse heart cytochrome *c* were added to a suspension of pseudomonad KB1 (equiv. 0.8 mg. dry wt.

organism/ml.) and OD (415) and OD (500) measured. Figure 3 is a plot of OD (415)-OD (500) against concentration of cytochrome *c* added; it will be seen that the curve is linear over a tenfold range of added cytochrome *c*.

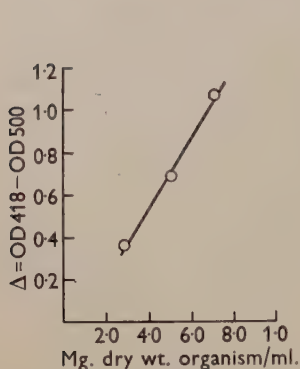


Fig. 2

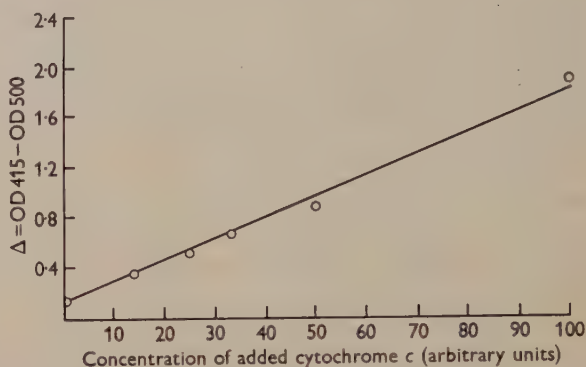


Fig. 3

Fig. 2. Estimation of cytochrome content of washed suspensions of pseudomonad KB1. Δ = optical density at wavelength of maximum absorption (418 m μ) minus optical density at wavelength of minimum absorption (500 m μ) as a function of concentration of organisms. Optical densities measured as for Fig. 1.

Fig. 3. Estimation of cytochrome content of washed suspensions of pseudomonad KB1. Proportionality of Δ (=absorption at Soret maximum of mammalian cytochrome *c* minus absorption at minimum) to concentration of added cytochrome *c* in the presence of washed suspensions of pseudomonad KB1 (equivalent to 0.8 mg. dry wt. organism/ml.). Optical densities measured as for Fig. 1.

Because it is uncertain whether the absorption at 418 m μ is due to a single cytochrome or to several, and because we do not know the extinction coefficients of the cytochromes of pseudomonad KB1, it is impossible to calculate from Δ the weight of cytochrome in a suspension. Consequently, all our results are given as optical densities and we propose to speak only of the relative cytochrome content of a suspension.

The relative cytochrome content of a suspension was measured as follows. Samples of culture were collected in a tube immersed in ice in a thermos flask. The sample was then filtered through a coarse fluted filter-paper, centrifuged and the deposit washed once in distilled water. The washed organisms were suspended in distilled water, the concentration measured and adjusted to equiv. 5.0 mg. dry wt. organism/ml. A sample was then placed in a 1 cm. cuvette, a few crystals of dithionite added and Δ was calculated from the OD measured at 418 and 500 m μ .

RESULTS

Growth in steady states

Growth was started by transferring 5-10 ml. of inoculum, prepared as described in the section on Methods, to 300 ml. of medium in the culture vessel, and allowing the growth to reach the maximum with full aeration but

no medium flow. Continuous culture was then begun by starting the medium flow and adjusting to the required rate. The growth of the culture was followed by frequent readings of the optical density. For each combination of the two variables studied (growth rate and air supply) the concentration of organisms would adjust itself to a practically steady value, and samples for examination of oxidizing capacity, cytochrome content and metabolic products were taken from such 'steady states'.

In order to study the effect of oxygen supply on the organism, we arranged conditions so that at any growth rate the carbon source and the air supply were alternately the growth-limiting factors. In the first case, air supply was in excess (as judged by the criteria enumerated in the Methods section) and the amount of organism was governed by the concentration of succinate. In order to reach the second state, the air supply was decreased, keeping the medium composition constant. As the oxygen supply was decreased, the growth rate of the organism fell to a value below that of the dilution rate, and the amount of organism in the culture decreased until the steady-state concentration, in the culture, of the growth-limiting factor again allowed the organism to grow at a rate which was equal to the dilution rate. In this way a new steady state was reached, in which oxygen was the growth-limiting factor. The actual quantities of organism achieved for the different air-limited states varied, but since all our measurements of oxidizing capacity, cytochrome content, etc., were referred to equiv. dry wt. of organisms and not to volume of culture, these variations did not affect the results.

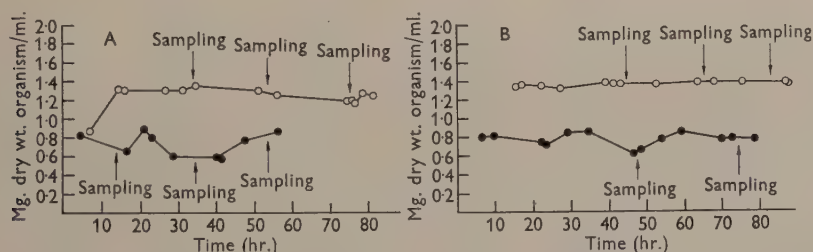


Fig. 4. Concentrations of pseudomonad KB1 growing in continuous culture under various steady-state conditions, as a function of time. (A): $\circ-\circ$, carbon-limited 'steady state'; dilution rate $D = 0.142 \text{ hr}^{-1}$; $\bullet-\bullet$, air-limited 'steady state'; dilution rate $D = 0.144 \text{ hr}^{-1}$; (B): $\circ-\circ$, carbon-limited 'steady state'; dilution rate $D = 0.228 \text{ hr}^{-1}$; $\bullet-\bullet$, air-limited 'steady state'; dilution rate $D = 0.216 \text{ hr}^{-1}$. The approximate times of sampling are indicated by the arrows on each graph. Measurement of pH values during each of the steady states showed these to be in the range 7.2–7.5 for the carbon-limited steady states, and in the range 6.6–6.9 for the air-limited steady states.

Figure 4 shows plots of concentration of organisms against time for a number of representative steady states. It can be seen that the variations in culture density were greater in the air-limited than in the carbon-limited steady states. This seems to be due to the fact that it is far easier to keep the concentration of carbon source constant, than to keep the air supply steady. In particular, we noticed that for a given setting, the actual speed of the stirrer motor often increased at night, and in the air-limited steady state the concentrations of

organisms in the culture tended to increase with it. We tried to overcome this by resetting the stirrer speeds late at nights, but were not wholly successful, because they tended to fluctuate again during the early hours of the morning. As far as possible we endeavoured to take samples only when the culture density was constant over several hours. The approximate times of sampling are shown on the graphs.

*Yield of organism with oxygen or succinate as
growth-limiting factor*

The range of pH values observed for the various steady states were from pH 7.2 to 7.5 for the carbon-limited steady states, and from pH 6.6 to 6.9 for the air-limited steady states. The relatively low pH values when air was the growth-limiting factor might be due either to incomplete breakdown of succinate with accumulation of acidic intermediates, or to succinate itself remaining in the medium.

Table 2. *Succinate consumed and intermediates found during growth of
pseudomonad KB1 in continuous culture*

Sample no.	Dilution rate (<i>D</i> (hr. ⁻¹))	Dry wt. organism (mg./ml.)	Succinate consumed (μ mole/ml.)	Steam volatile acids found (μ equiv./mg. dry wt.)	Keto-acids found (μ mole/mg. dry wt.)	Yield constant (mg. organism/ mg. succinate consumed)
Carbon-limited states						
A10	0.082	1.09	34.8	0.05	0.16	0.27
A21	0.142	1.31	34.7	0.28	0.06	0.32
A21	0.142	1.30	35.0	0.36	0.07	0.32
A22	0.142	1.25	35.0	0.17	0.03	0.30
A17	0.228	1.38	34.9	0.14	0.04	0.34
A7	0.314	1.70	34.2	—	0.15	0.42
A26	0.401	1.81	34.6	—	0.14	0.44
A27	0.401	1.53	34.3	—	0.18	0.38
Air-limited states						
A11	0.072	0.87	26.0	0.03	0.22	0.28
A23	0.144	0.77	20.3	—	0.07	0.32
A24	0.144	0.96	27.2	0.02	0.29	0.30
A18	0.216	0.84	19.7	0.04	0.09	0.36
A19	0.216	0.66	15.0	0.27	0.16	0.37
A8	0.293	1.12	23.3	0.19	0.28	0.41
A9	0.293	0.96	20.0	0.40	0.25	0.41
A28	0.383	0.42	10.3	0	0.40	0.35
A29	0.383	0.70	15.6	0	0.21	0.38

Table 2 shows the amounts of succinate consumed, the steam-volatile and keto-acids which accumulated and the yield constants (=mass of organisms formed/unit mass of succinate consumed) at different growth rates for the carbon-limited and air-limited steady states. It can be seen that for both the growth-limiting factors, the yield constants were practically the same, and that no significant amounts of steam volatile and keto-acids accumulated in the growth medium. Although under certain conditions pseudomonad KB1 can accumulate partially oxidized products from succinate (Kogut & Podoski,

1953), this does not appear to happen when oxygen concentration becomes growth limiting. Given growth-limiting concentrations of oxygen, it appears that part of the succinate is oxidized to the same extent as when oxygen is in excess, whilst part is not attacked at all.

*Variation in oxidizing capacity of organisms with
limiting factor and growth rate*

Measurement of the oxidizing capacities of organisms (expressed as Q_{O_2} , succinate) were carried out on two or three samples from each steady state. Figure 5 shows these values plotted against dilution rate.

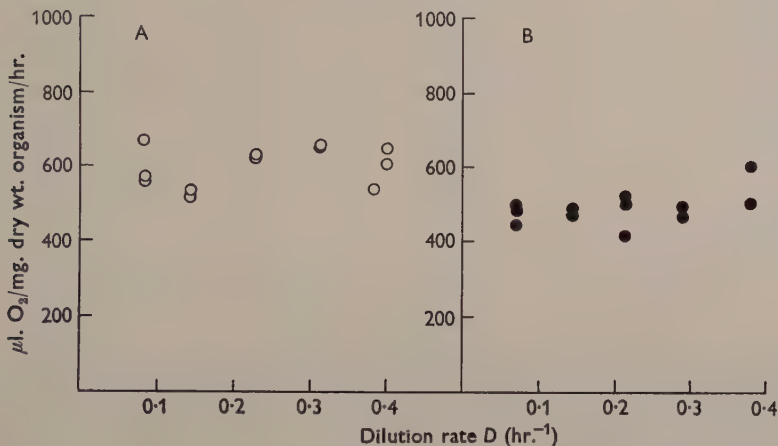


Fig. 5. Oxidizing capacity, measured as Q_{O_2} (succinate) of washed suspensions of pseudomonad KB1 grown under different conditions, as a function of dilution rate. Each circle represents the mean of duplicate determinations carried out on a separate sample of organisms grown under the indicated conditions at the indicated rate. The separate samples for each set of growth conditions were taken at intervals of several hours. Endogenous values, which were never more than 5% of the substrate respiration rates, have been ignored.

Warburg cups contained organisms 1–2 mg. dry wt.; 30 μ mole phosphate buffer (pH 7.4); 50 μ mole sodium succinate in total volume 2.5 ml. The centre wells contained strips of folded filter-paper moistened with 10% (w/v) KOH. Gas phase air. Measurements were made at 30° and were started by tipping the suspensions from the side arms. Readings were taken over the period 5–35 min. after tipping, when the oxygen uptake was linear with time; the Q_{O_2} values were calculated from these. A: organisms grown with succinate as growth-limiting factor; B: organisms grown with air as growth-limiting factor.

Each point represents the measurement, carried out in duplicate, on one separate sample from a given steady state. Statistical analysis of the values obtained from the carbon-limited and air-limited steady states by the *t*-test shows that the differences are significant at the 0.1% level. On the other hand, the product-moment coefficients of correlation between Q_{O_2} (succinate) and dilution rate for either of the two groups were not significant at the 5% level; there were insufficient data to determine whether the rise in Q_{O_2} at the fastest dilution rate observed in the air-limited condition was significant. It

might be argued that for any given growth rate there must be a corresponding minimum respiration rate since the organism is an obligate aerobe. Pirt (1957) has shown that the oxygen uptake rate of populations of organisms growing in a steady state in continuous culture can be calculated from the dilution rate, the amount of substrate utilized and the 'oxygen demand constant' P =mole oxygen consumed/mole substrate utilized. Washed suspensions of pseudomonad KB1, harvested in the logarithmic phase of

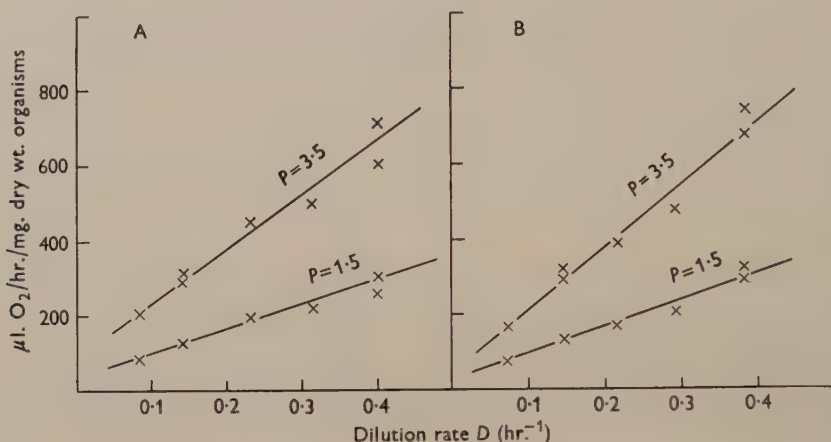


Fig. 6. Calculated oxygen uptake rates of pseudomonad KB1 growing in continuous culture with succinate as sole source of carbon. The values for oxygen uptake rates in $\mu\text{l.}/\text{hr.}/\text{mg.}$ dry wt. organism have been calculated according to the equation of Pirt (1957)

$$\frac{-dc}{dt} = PD(s_0 - s),$$

where P =oxygen demand constant (mole oxygen consumed/mole substrate utilized), D =dilution rate, and $s_0 - s$ =amount of substrate consumed. The oxygen uptake rates have been calculated for complete oxidation of succinate ($P=3.5$) and for partial oxidation of succinate by washed suspensions of pseudomonad KB1 according to Kogut (1952) ($P=1.5$). A: organisms grown with succinate as growth-limiting factor; B: organisms grown with air as growth-limiting factor.

growth, oxidize succinate with the consumption of 1.5 mole oxygen/mole succinate (Kogut, 1952; Kogut & Podoski, 1953). Unfortunately, we do not know the oxygen demand constant for succinate utilization by growing cultures of pseudomonad KB1. However, we have calculated the oxygen uptake rates in $\mu\text{l.}/\text{hr.}/\text{mg.}$ dry wt. organism for pseudomonad KB1 growing in continuous culture at the different growth rates used, assuming complete oxidation of succinate ($P=3.5$) and for $P=1.5$. These values are shown in Figs. 6a and 6b.

It can be seen from a comparison of Figs. 5 and 6 that at low dilution rates the Q_{O_2} values of washed suspensions are in excess of the calculated oxygen uptake rates of the growing organisms. At the higher dilution rates, the oxygen uptake values of the growing organisms calculated on the assumption that $P=3.5$ correspond fairly closely to the measured Q_{O_2} values of washed suspensions.

Variation in relative cytochrome content of organisms with limiting factor and growth rate

The relative cytochrome content of organisms grown under conditions where either succinate or air supply were the growth-limiting factors, and at different growth rates, was determined as described in the section on Methods. Figure 7 shows the values obtained plotted against dilution rate. Two or three samples were taken from each steady state and each point represents the value obtained for a single separate sample.

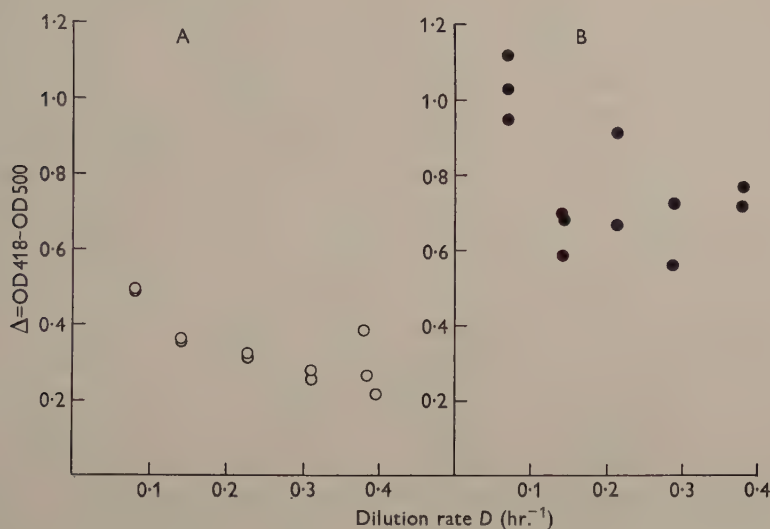


Fig. 7. 'Relative cytochrome content', measured as $\Delta = \text{OD at } 418 \text{ m}\mu \text{ minus OD at } 500 \text{ m}\mu$ of washed suspensions of pseudomonad KB1 at constant concentrations (equivalent to 5.0 mg. dry wt. organism/ml.) as a function of dilution rate. Each point represents the measurement, carried out on a separate sample of organisms grown under the indicated conditions. Separate samples for each set of growth conditions were taken at intervals of several hours. Measurements were carried out on washed organisms suspended in water containing a few crystals of sodium dithionite, in a 1 cm. cuvette, placed in a quartz prism Hilger Uvispec Spectrophotometer, fitted with a diffusion screen of Whatman No. 3 filter-paper, soaked in medicinal paraffin, between the cuvettes and the photocell. A: organisms grown with succinate as growth-limiting factor; B: organisms grown with air as growth-limiting factor.

Statistical analysis of the relation between relative cytochrome content and dilution rate shows a product-moment correlation of -0.783 , which is significant at the 1 % level, for the carbon-limited states and a correlation of -0.517 , which is barely significant at the 5 % level, for the air-limited steady states. Thus, the relative cytochrome content tended to decrease with increasing growth rate. At all the growth rates used, the relative cytochrome content of organisms which had been grown under air-limited conditions was more than double that of organisms grown with air supply in excess.

DISCUSSION

It would appear that with pseudomonad KB1, growth under conditions of limiting air supply, at a low rate, gives organisms with increased light absorption typical of cytochromes. Comparing changes at the same growth rates for the two different factors which limited growth, it further appears that decrease of the air supply alone is sufficient to bring about increased light absorption. It is not possible, unfortunately, to determine by the above technique whether this increased absorption can be ascribed to any particular cytochrome component. Washed suspensions and crude extracts of this organism showed two broad absorption bands in the α -region, at 551–556 m μ and at 560–565 m μ , and only one (unsymmetrical) peak in the Soret region (see Fig. 1) which probably represents the Soret bands of at least two cytochromes, one of which was isolated and purified by Kogut (1957). This cytochrome has its reduced absorption peak located at 417 m μ . What cannot be determined is the quantitative composition of the cytochrome system, since the specific extinction coefficients for the various cytochromes are not known. It is, therefore, also impossible to decide whether an increase in light absorption with different growth conditions represents an increase in the total amount of cytochromes produced, or a change to a predominant cytochrome with higher extinction coefficient.

A second question which cannot be answered by the present work is whether the change in relative cytochrome content is either a phenotypic change, or is due to the selection of variants. In these experiments we were particularly interested in establishing the connexion between cytochrome content and growth rate, physiological state of the organism and oxygen supply. Data were, therefore, obtained for conditions in steady states, and not for the transition periods before the steady states were reached. Moss (1956), with *Aerobacter aerogenes*, found increases in cytochromes a_2 and a_1 as the oxygen concentration was decreased from 10^{-3} to 10^{-6} M, and these changes could not be accounted for by selection of variants. Moss made his observations from the time of inoculation, over a total growth period, for any one oxygen concentration, of not more than 9 hr.; 'in no case was cytochrome increased after 5 hr.'.

Our findings show general agreement with the data of Moss (1956) for *Aerobacter aerogenes*. He found that when this organism was grown with different steady-state concentrations of oxygen in continuous culture (the steady states being of less than 9 hr. duration) the content of cytochrome a_2 in the organism increased as the oxygen concentration decreased from 10^{-3} to 10^{-6} M, whilst the cytochrome b_1 content remained constant. As the oxygen concentration was decreased further from 10^{-6} to 10^{-9} M, the concentration of cytochrome a_2 and b_1 in the organisms fell. The absorption due to cytochrome a_1 (which was always small relative to the absorption due to the other cytochromes) showed a slight increase with decreasing oxygen concentration over the range 10^{-3} to 10^{-6} M. At the same time, the growth rates of *A. aerogenes* in Moss's experiment also varied with the oxygen concentration in the medium. *A. aerogenes* is, of course, a facultative aerobe, with a respiratory and cytochrome system very different from that of pseudomonad KB1. In fact, the

only component of the pseudomonad KB1 cytochrome spectrum similar to one seen in *A. aerogenes* is the α -band at 560 m μ which Moss ascribes to cytochrome b_1 . But even this similarity in spectroscopic properties does not mean that the same substance is present in the two different organisms.

Observations on the production of cytochrome in *Pseudomonas fluorescens* have been reported by Lenhoff & Kaplan (1953), Lenhoff (1954) and Lenhoff *et al.* (1956). They found that when their organism was grown in batch culture without aeration or agitation, it produced much more of a cytochrome spectroscopically similar to animal cytochrome c , and a cytochrome peroxidase which could not be separated from it, than it did when grown with aeration. Their medium contained 0.5 % (w/v) sodium nitrate (in addition to 0.4 % of Difco Yeast Extract), so that anaerobic growth might have occurred at the expense of nitrate reduction. It is known from the work of Baalsrud & Baalsrud (1954) and of Sato & Egami (1949) that high values of cytochrome content are associated with growth under such conditions, i.e. with the development and operation of electron transport mechanisms in which nitrate is the final acceptor. In other words, this might be regarded as a special instance of a facultative system in which a cytochrome is part of a particular electron transport mechanism which may be inducible by its final electron acceptor, in this case nitrate. Other cases in which the production of cytochromes appears to be induced by the final electron acceptor are those observed by Chin (1950) and by Ephrussi & Slonimski (1951) where the cytochromes a , b and c of yeast appeared in response to aeration. Pseudomonad KB1 does not show any nitrate-reductase activity and does not grow under anaerobic conditions, either with or without nitrate (unpublished observations). The mechanism by which its relative cytochrome content is increased when it is grown on a simple defined medium with oxygen as the growth-limiting factor may therefore be an entirely different one.

Increase in haem respiratory pigments under conditions of low environmental oxygen concentration appears to be a general phenomenon and can occur in cases where selection of variants must be ruled out (Moss, 1956). Moreover, it has been shown by Moss that it can occur in a system (the cytochrome a_2 of *Aerobacter aerogenes*) which also shows an inductive response of increased cytochrome content to increased oxygen, over a different, lower range. Possible mechanisms to account for the apparent response of increased synthesis to decreased oxygen concentrations have been suggested by various authors (Moss, 1956; Lenhoff *et al.* 1956). Under the conditions of our experiment, the question of the possible effect of oxygen concentration on cytochrome content can be approached in the following way.

Longmuir (1954) has shown that the relation of the respiration rates of bacteria to oxygen concentration can be approximately described by the Michaelis-Menten equation. If it is assumed that a certain fixed fraction of the maximum possible respiration rate is required to allow bacteria to grow aerobically at a given rate, then it follows from Longmuir's data that for growth at any given rate, there exists a definite, minimum steady-state oxygen concentration. Under the experimental conditions here reported, the growth

rates were controlled by the rates of medium flow. On the other hand, in such a system, the growth rate is a function of the steady-state concentration, in the culture of the growth-limiting factor. It follows from the theory of continuous culture (Herbert, Elsworth & Telling, 1956) that under the air-limited conditions, the steady-state concentrations of oxygen in the culture must have been exactly those which allow the organisms to grow at rates equal to the dilution rates with all other nutrients, including carbon source, in excess. In the carbon-limited steady states, on the other hand, the oxygen concentrations in the culture must have been in excess of those required to allow growth at the given rates. It might be asked, therefore, 'can excess of carbon source stimulate cytochrome formation, or can excess of oxygen, either directly or indirectly inhibit cytochrome production?' A possible mechanism of inhibition by excess oxygen might be the production of an inhibitory substance or substances by an alternative electron transport system functioning at high oxygen concentrations. Such a changeover of the respiratory mechanism to an alternative pathway, possibly of a flavin nature, has been suggested by Lenhoff *et al.* (1956). Herbert (1955) has shown that inhibition due to peroxide formation in the medium under aerobic conditions accounted for the failure of small inocula of *Pasteurella pestis* to grow aerobically on agar plates in the absence of haematin or catalase.

The negative correlation between relative cytochrome content and growth rate in the carbon-limited steady states would follow from the assumption that the rate of cytochrome synthesis in these was determined by a factor—such as oxygen concentration—not governing the rate of general cell synthesis; variation of the latter, i.e. growth rate would, then, alter the relative cytochrome content of organisms in the observed direction. An alternative mechanism for the control of the rate of cytochrome synthesis might be the degree of oxidation of the electron-carrier system, in a manner analogous to the self-regulatory mechanism for the synthesis of photosynthetic pigments in *Athiorhodaceae* suggested by Stanier & Cohen-Bazire (1957).

We wish to express our sincere gratitude to Dr S. R. Elsdon for his constant encouragement, criticism and advice; and to Mr J. W. Lightbown for many helpful discussions. We would also like to thank Miss M. V. Mussett for help and advice with the statistical analysis; and Miss Jennifer Bishop and Miss Felicity Westmoreland for willing and conscientious technical assistance.

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Electron Microscope Studies of HeLa Cells Infected with Herpes Virus

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SUMMARY: Monolayer cultures of HeLa cells were exposed to a high multiplicity of herpes virus per cell. Samples of cells taken at successive time intervals after infection were sectioned and examined by electron microscopy for characteristic ringed particles. Replicate cultures of cells were assayed for infective virus. Despite an estimated input of 6 HeLa plaque-forming units (pfu)/cell, an infective centre count indicated that only 6% of the cells yielded virus. No increase in infective virus was found in the cultures at 9 hr. and no characteristic particles were found in sections of 28 cells. At 12 hr. new virus appeared in the cell fraction and particles were found in the nuclei of 2 of 25 cells. At 26 hr. after infection and later, there was a large increase in virus in both cell fraction and medium, and large numbers of particles were present in the nucleus and cytoplasm and on the cell surface.

The electron microscope studies of Morgan, Ellison, Rose & Moore (1954) on sections of chorioallantoic membrane cells infected with herpes virus have shown particles in the nucleus and cytoplasm of the infected cells. The size of the particles in the cytoplasm agrees with other estimates, and it could be tentatively assumed that they were in fact virus particles. Such particles were round or oval in section and had a characteristic internal structure. The intracellular cytoplasmic particles consisted of a dense central body, surrounded by a double membrane. In the nucleus, however, the particles contained the same central body but only one membrane.

A study of the growth of herpes virus in HeLa cells has shown that formation of a new infective virus is first detectable in the cells at 12 hr. after exposure. The virus is not released into the culture medium, however, until 16 hr. or more after exposure (Stoker & Ross, 1958). It has also been found that the cells start to synthesize excess deoxyribosenucleic acid (DNA) 6-9 hr. after infection, before the appearance of infective virus (Newton & Stoker, 1958). It was, therefore, of interest to examine HeLa cells by electron microscopy during the latent period to see whether virus particles appeared at the time of DNA synthesis but before the rise in infectivity. It was hoped that their fairly large size and characteristic cross-section might make these particles relatively easy to identify amongst the other constituents of nucleus and cytoplasm.

METHODS

The maintenance of the HeLa cells, the culture media, and certain other experimental details will be described more fully in a separate communication on the growth of herpes virus (Stoker & Ross, 1958).

The Melbourne egg- and mouse-adapted variant of the HF strain of herpes virus (HFEM) was used. Seed suspensions were prepared from infected HeLa cell culture media after eight or more passages in these cells. The virus was assayed by pock counts on the chorioallantoic membrane (CAM) because this was more than ten times more sensitive than titration in HeLa cells, even after passage in these cells. Virus input, however, has been deduced from adsorption data obtained by Farnham (1958), using a plaque-counting method in HeLa cells.

Flat-sided tubes containing monolayers of 10^5 HeLa cells were exposed to 200 pock-forming units of virus/cell. This is equivalent to 7 HeLa plaque-forming units/cell under the same experimental conditions. After 2 hr. the excess virus was removed by washing and treatment with herpes antiserum, and the cells were then incubated at 37° in medium. Two hours allows adsorption of about 80 % of HeLa infectious virus, so the theoretical input multiplicity was approximately 6. At 5, 9, 12 and 26 hr. the medium was removed from 4 tubes, pooled, and titrated for infective virus. The cells from the tubes were removed from the glass with a 1/20,000, w/v, solution of sodium EDTA (ethylenediaminetetra-acetic acid), pooled, pipetted to break up clumps, and counted in a haemocytometer chamber. The pooled cell suspension was then centrifuged and the supernatant EDTA solution removed for virus assay.

The cell pellet was washed once with buffered saline, redeposited and left in 1 % (w/v) osmium tetroxide buffered at pH 7.4 for 40 min. The fixative was then removed and the cells replaced in buffered saline for not more than 2 hr. before embedding. For embedding, a mixture of 8 parts butyl methacrylate to 2 parts methyl methacrylate was used. Sections were cut on a Cook and Perkins ultra-microtome using a glass knife. The electron micrographs were taken on a Siemens Elmiskop I microscope at 60 kV. Uninfected HeLa cells, maintained in medium and prepared in the same way, were also fixed for sectioning. During the latent period, at 5 and 9 hr., unfixed cells, suitably diluted in medium, were inoculated on to the chorioallantoic membrane of embryonated eggs for an estimation of the proportion of pock producing, i.e. infected, cells.

RESULTS

The time of appearance of infective virus in the cultures is shown in Fig. 1. Less than 0.01 % of the original inoculum was present in the fluid and EDTA fractions after washing and treating the cells with antiserum. Despite the large input of virus only 5×10^3 cells in each tube yielded virus when inoculated on to chorioallantoic membrane, that is 6 % of the total cells. A rise in infectivity was first detected in the EDTA fraction at 12 hr. but there was no appearance of new virus in the medium at this stage. At 26 hr. the EDTA fraction and medium showed a considerable rise in infective virus. These results agree closely with more detailed investigations of the growth of virus in HeLa cells which also show that the rise in the EDTA fraction coincides with the increase in infective virus in the disintegrated cells (Stoker & Ross, unpublished).

Electron microscopic appearances

Sections of normal HeLa cells show densely granular material in the nucleus and recognizable nucleoli. A few cells, however, contain rather empty nuclei resembling those in infected cells (see below) but none of the typical ringed particles are seen, and there is no accumulation of the granular material at the nuclear membrane in uninfected cultures.

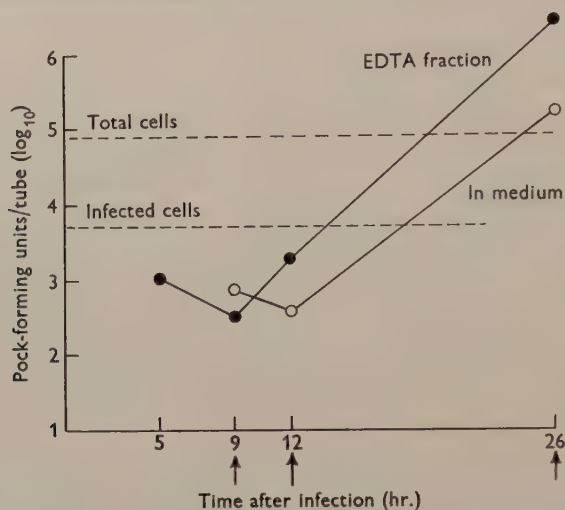


Fig. 1. Development of infective herpes virus in medium and EDTA fraction of HeLa cells inoculated with 6 plaque-forming units/cell. Arrows denote times at which cells were fixed for examination by electron microscopy.

At 9 hr. after infection (Pl. 1, fig. 1) no typical ringed particles were seen in cytoplasm or nucleus in sections of 28 cells taken at random. The nuclei were not obviously different from the normal cells, but irregular material, elongated, oval or rounded in section, was present on or near the cell surface. This material did not resemble the ringed particles present later in the infection, and they were taken to be cytoplasmic protrusions.

At 12 hr. after infection, examination of sections of 25 cells showed two cells with 9 and 4, respectively, typical ringed particles visible in the nuclei (Pl. 2, figs. 2, 3). These nuclei also showed considerable loss of granular material except at the nuclear margin. Four other cells exhibited loss of nuclear structure without recognizable ringed particles. The remaining cells appeared normal.

At 26 hr. after infection many of the nuclei were almost devoid of granular material, and large numbers of typical ringed particles were seen (Pl. 3, figs. 4, 5; Pl. 4, figs. 6, 7). These were present in the nucleus, in the cytoplasm and on the cell surface. In these, as in the 12 hr. cultures, it was not possible to tell what proportion of the cells contained particles, because each cell could not be serially sectioned and examined in all its dimensions.

In a separate experiment carried out in the same general way, cells were

taken 96 hr. after exposure to a low input of virus (0.1 pock units/cell), when stained preparation showed gross cytopathic change (Ross & Orlans, 1958). Electron microscopic examination of sections (Pl. 5, fig. 8; Pl. 6, fig. 9; Pl. 7, fig. 10) again showed extensive loss of nuclear structure, and large numbers of ringed particles, mostly in the nuclei and on the cell surface, rather than in the cytoplasm.

General features of infected cells

In the 26 and in the 96 hr. specimens particles in the nucleus showed a central dense granule and single membrane, while those in the cytoplasm and on the cell surface apparently had double membranes. It is possible, however, that this effect might be caused by a thickening of the single membrane, because the gap between the two membranes is itself granular and not so clear as the area between the inner ring and central spot.

The largest diameter of 31 of the extranuclear particles sectioned nearly equatorially through the central body, gave a mean value of 135 m μ . Particles were sometimes seen with thick or double membranes in the marginal granular material of the nucleus (Pl. 5, fig. 8), but these might have been in cytoplasmic invaginations of the nuclear membrane. An occasional particle with a double ring appeared to be within the nuclear membrane, however (Pl. 3, fig. 4). Most of the nuclear particles were scattered and only one area was seen which might constitute a colony of developing virus.

Whereas the extranuclear virus at 26 hr. was in the cytoplasm, at 96 hr. (in a different experiment with a small virus input), it was found largely on the cell surface, or in complicated crypts and invaginations of the cytoplasmic membrane (Pl. 6, fig. 9). Apparently treatment of the cells with EDTA, which removes calcium and magnesium ions, does not release all the virus from the surface of the cells.

DISCUSSION

To evaluate the electron microscopic changes during virus growth it is desirable to know the stage of infection in each cell examined, or alternatively to examine a population of cells which were all infected simultaneously, with subsequent events in the growth cycle proceeding synchronously. Except with bacteriophage, this ideal has not been achieved. In the examination of HeLa cells infected with herpes virus reported in this paper only 6% of the initially infected cells were shown to yield virus, by infective centre counts on chorioallantoic membrane. Calculation of virus input suggests that this is a low estimate, similar to that found by Kaplan (1957) with herpes virus in rabbit kidney cells. It is known that new infective virus can be detected in the cells and EDTA fraction at 12 hr. but not at 9 hr. In the sections characteristic particles were also found first at 12 hr. in 2 of 25 cells examined. No particles were found at 9 hr., but it would be impossible to examine a large enough number of cells sufficiently completely to exclude the presence of such particles. Nevertheless, it is known that the cells at this stage have synthesized an excess of DNA (Newton & Stoker, 1958). If herpes virus itself

contains DNA it would require, even at a conservative estimate, an average of 10^5 to 10^6 particles/cell to account completely for the excess at 9 hr. There was certainly no evidence from the electron micrographs at 9 or even 12 hr. that this number of particles was present.

We are grateful to Miss S. Vernon Smith and Mr G. J. Hills for the electron microscopy. This work was supported by grants from the Medical Research Council and the Agricultural Research Council.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. 9 hr. after infection. Apparently normal HeLa cell. $\times 12,000$.

PLATE 2

Fig. 2. 12 hr. after infection. Part of nucleus with the first characteristic single ringed particles to be seen. (In this and other plates some of the particles are indicated with arrows.) $\times 65,000$.

Fig. 3. 12 hr. after infection. Part of nucleus and cytoplasm of another cell, showing characteristic particles. $\times 33,000$.

PLATE 3

Fig. 4. 26 hr. after infection. Giant cell showing part of two nuclei. Typical single ringed particles in nucleus and double ringed particles in cytoplasm. One intranuclear particle has a double ring (arrow). $\times 33,750$.

Fig. 5. 26 hr. after infection. Part of three nuclei in giant cell with intranuclear, single ringed particles. $\times 31,500$.

PLATE 4

Fig. 6. 26 hr. after infection. Nucleus and disrupted cytoplasm of cell with characteristic particles. $\times 31,500$.

Fig. 7. 26 hr. after infection. Cytoplasmic particles lying inside membranes, possibly invaginations of cell membrane. $\times 31,500$.

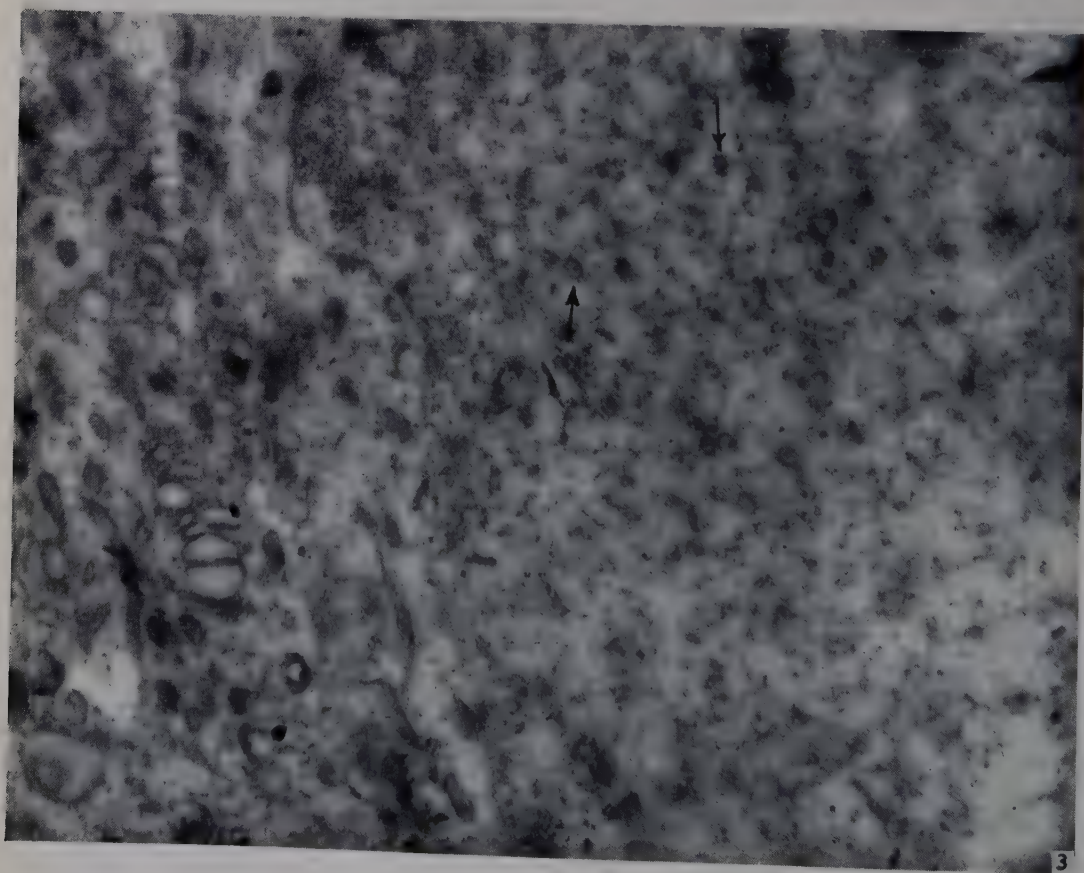
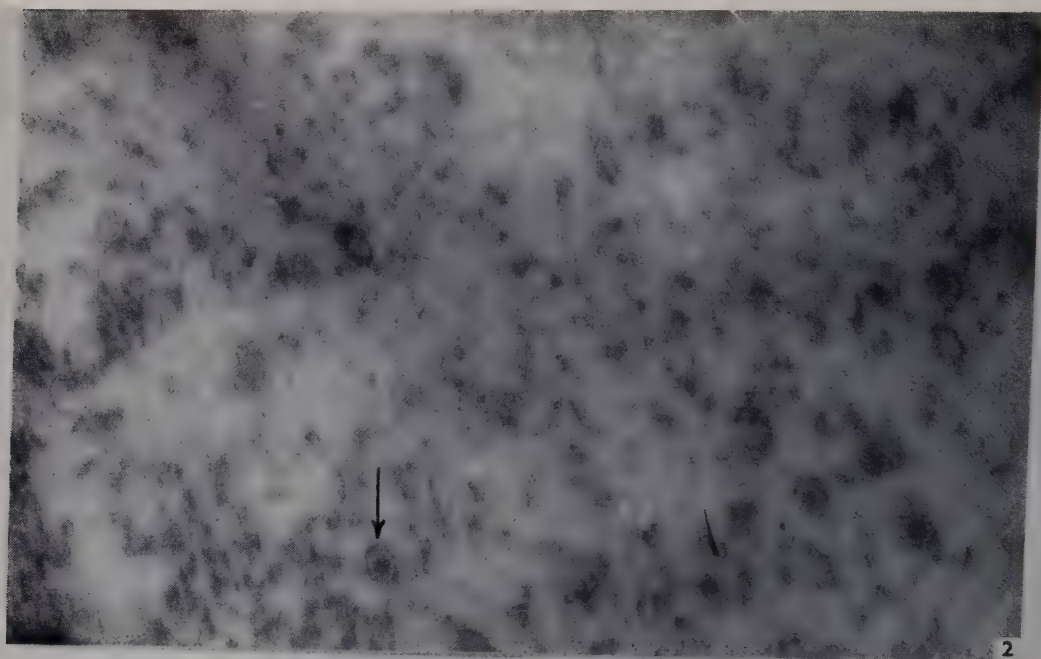
PLATE 5

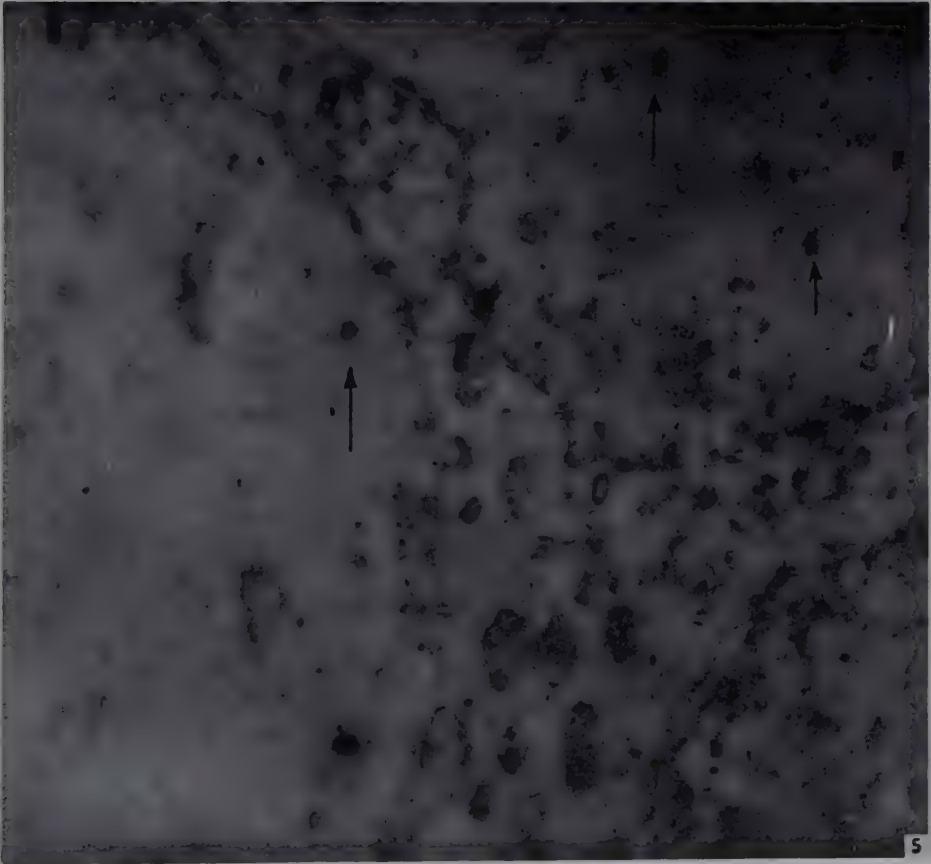
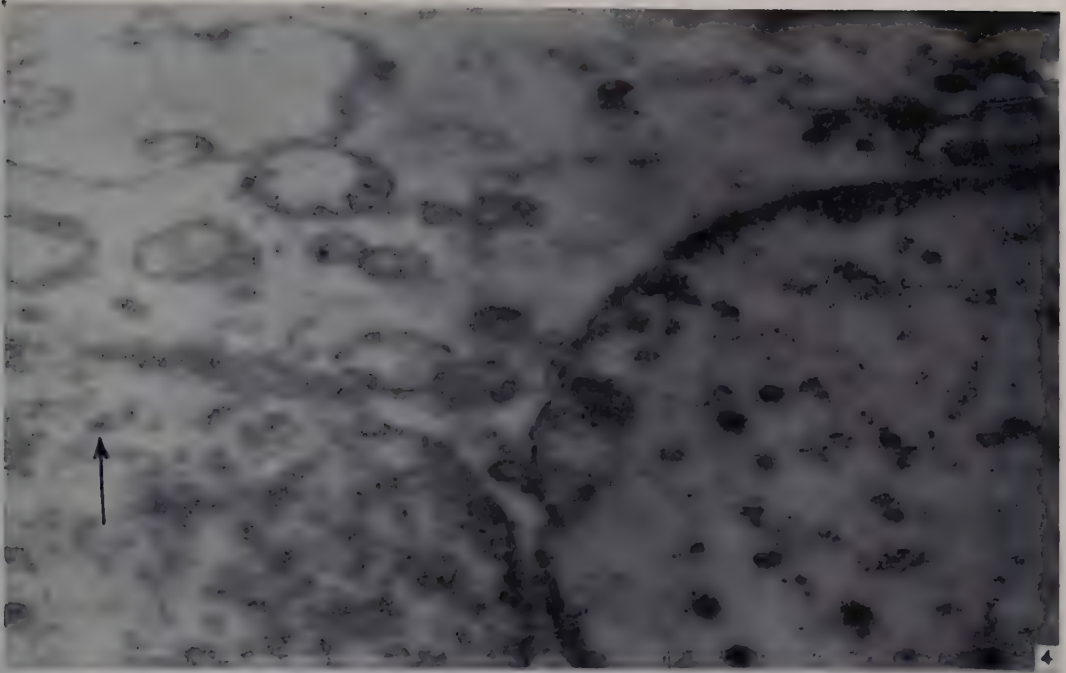
Fig. 8. 96 hr. after infection. Characteristic particles in nucleus, cytoplasm and on cell surface. Group of double ringed particles are shown inside the nucleus, possibly in invaginated nuclear membrane. $\times 32,400$.

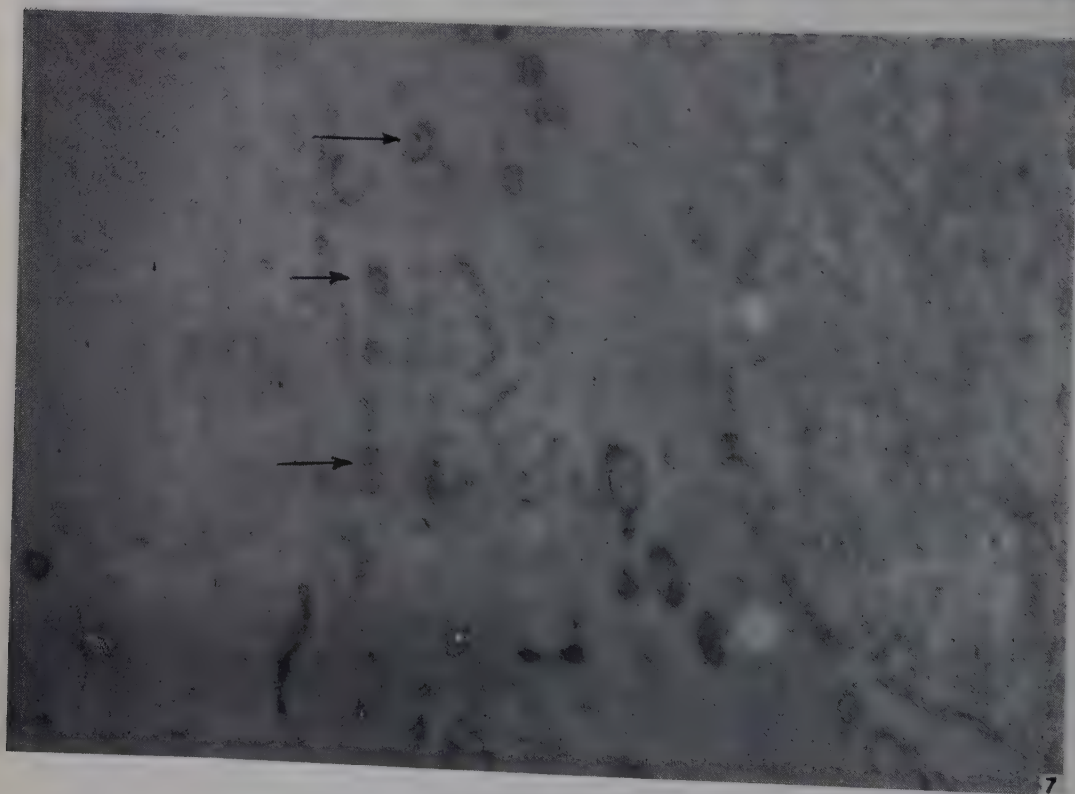
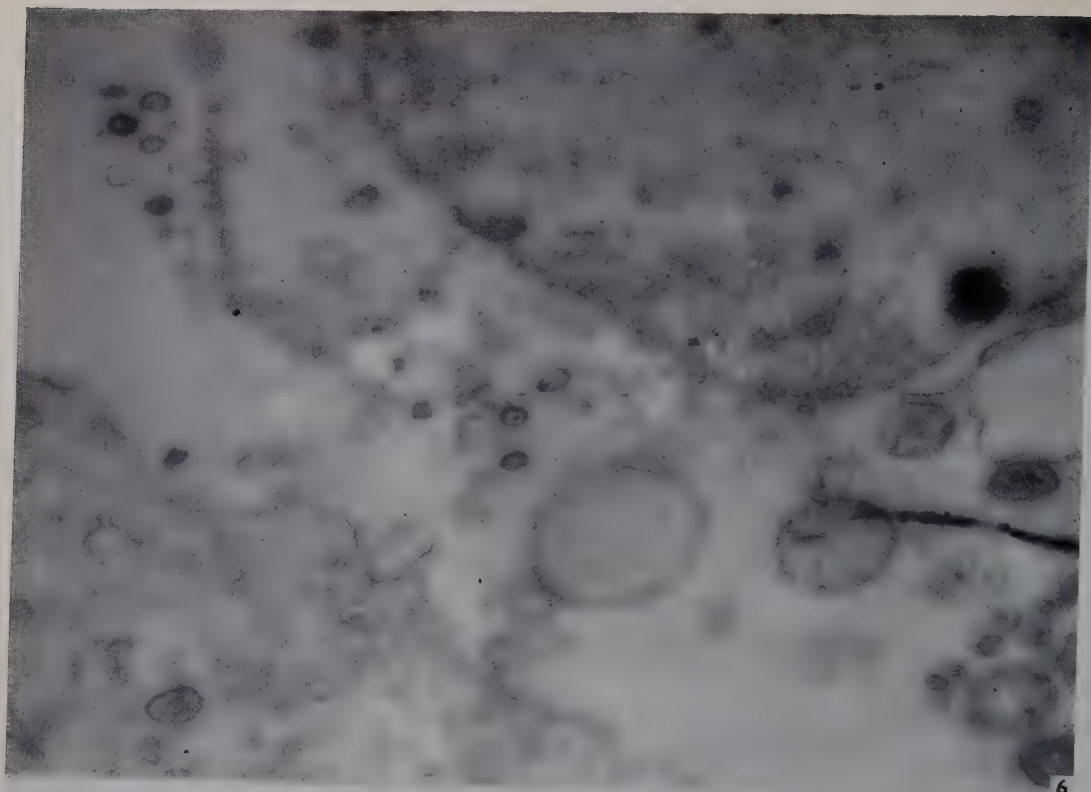


M. G. P. STOKER, K. M. SMITH & R. W. ROSS—ELECTRON MICROSCOPY OF HERPES
VIRUS. PLATE 1

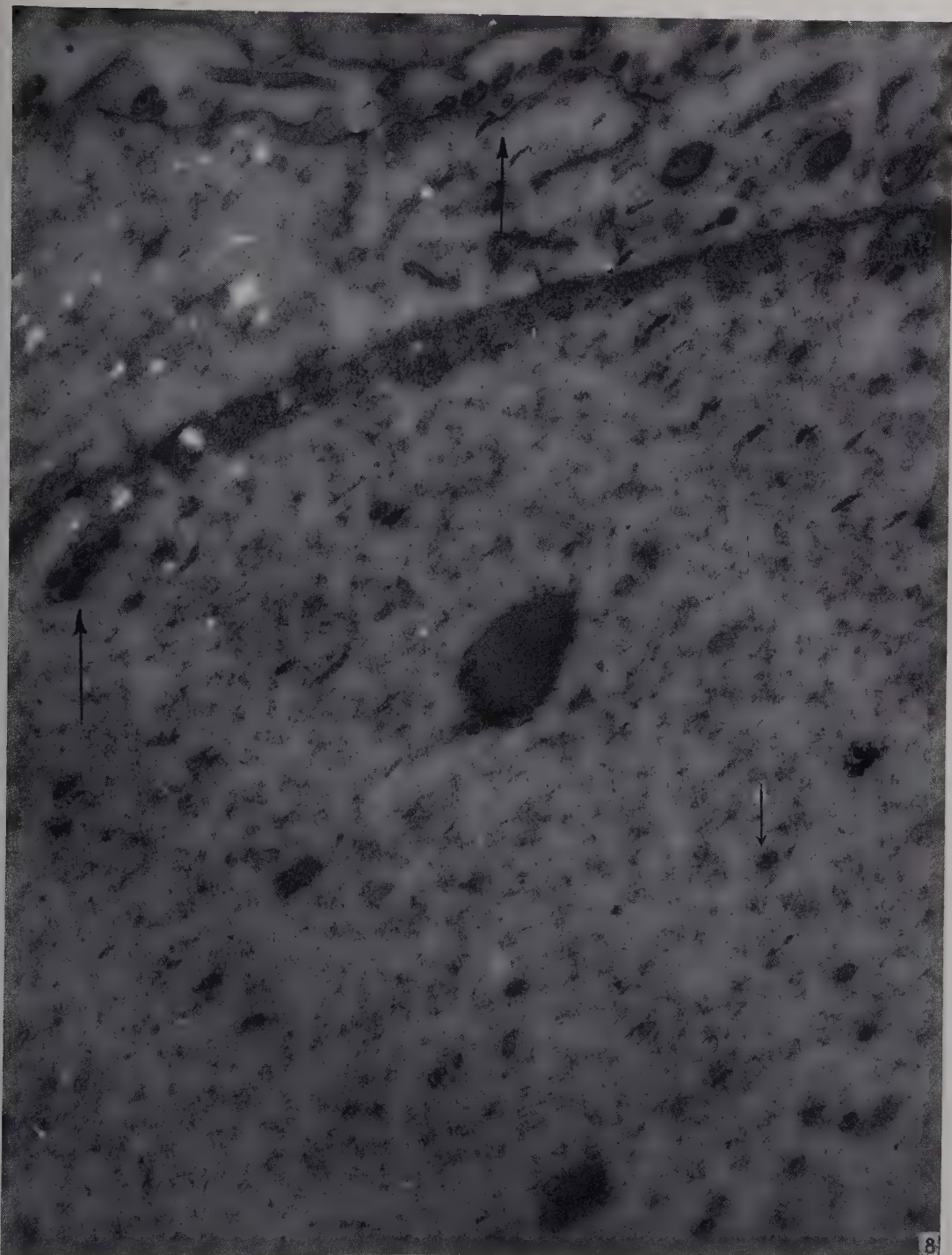
(Facing p. 248)

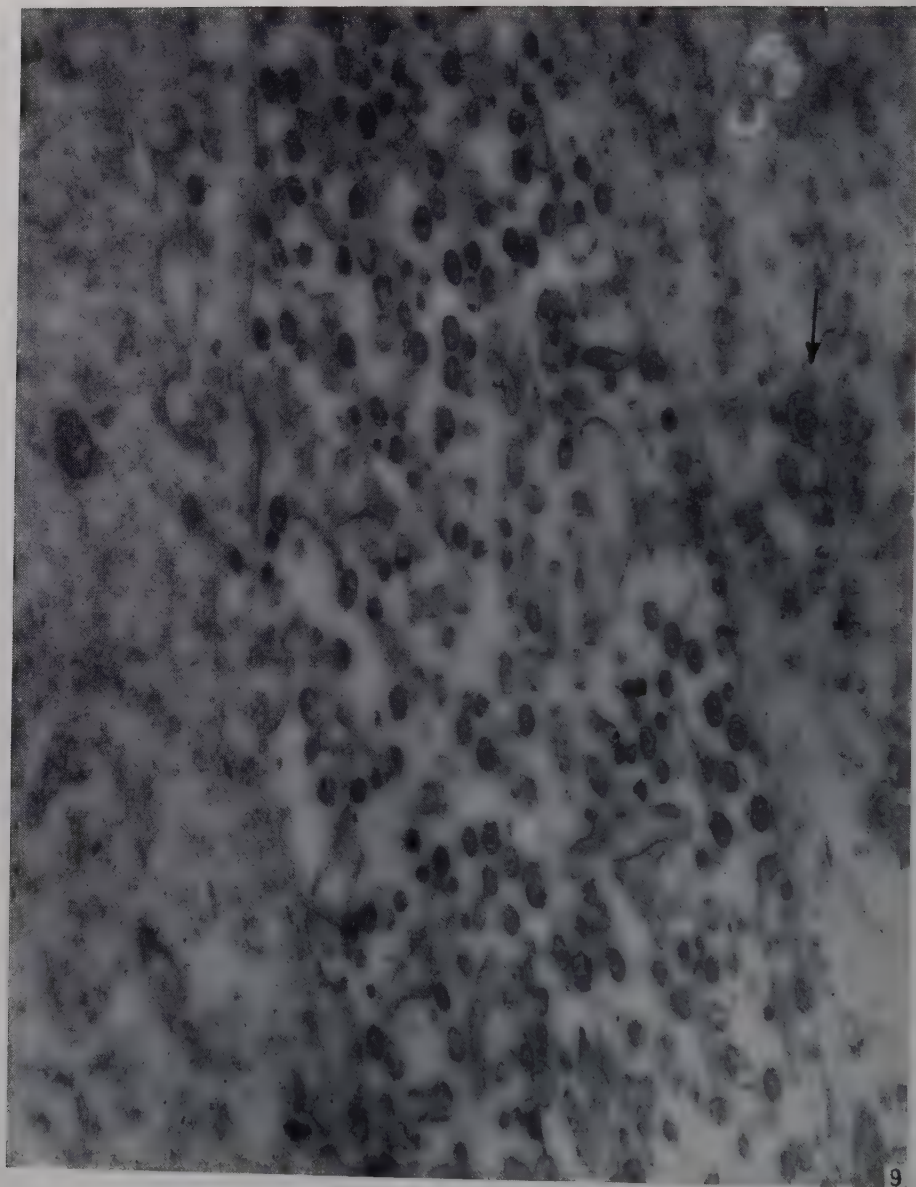






M. G. P. STOKER, K. M. SMITH & R. W. ROSS—ELECTRON MICROSCOPY OF HERPES
VIRUS. PLATE 4





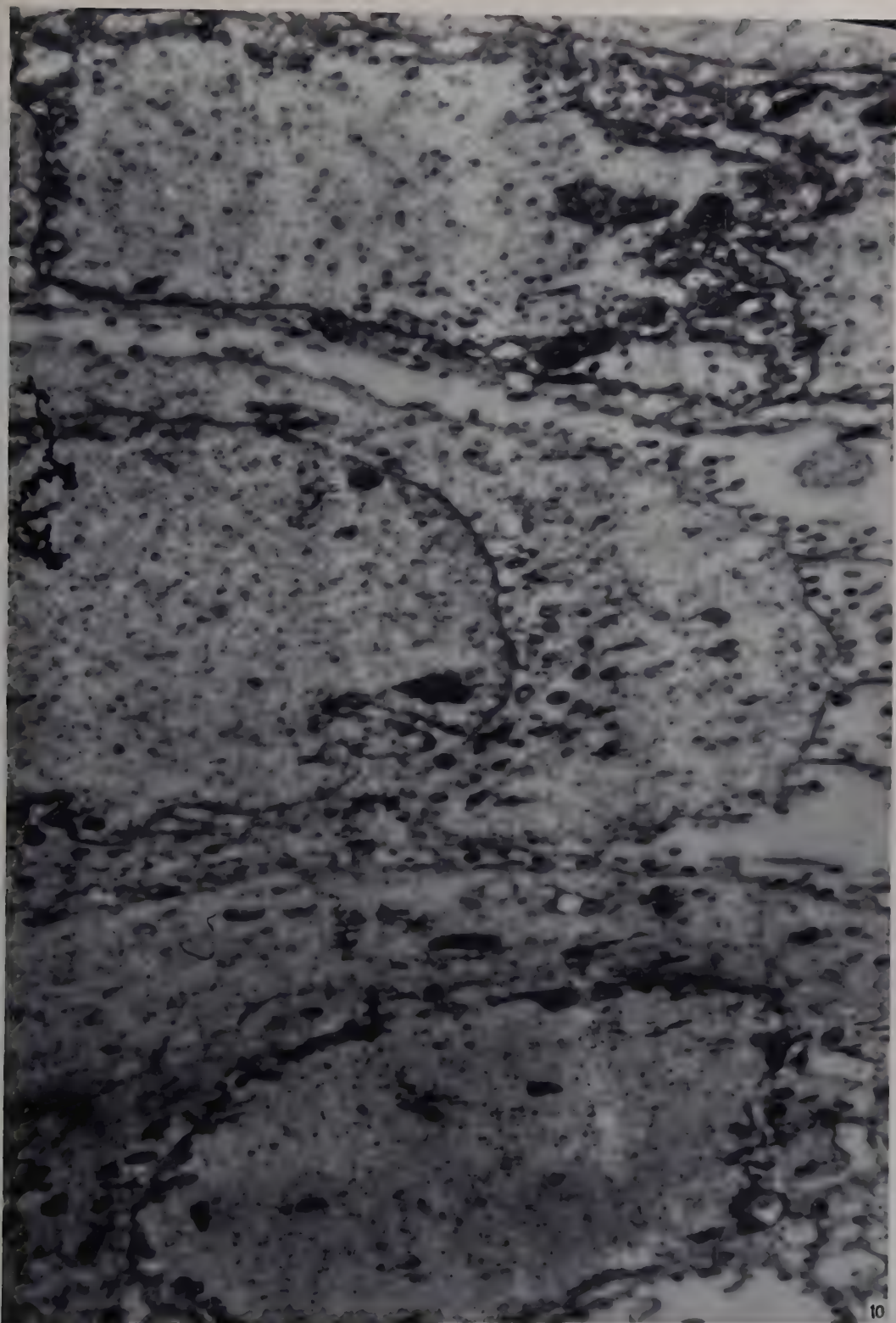


PLATE 6

Fig. 9. 96 hr. after infection. Space between two cells packed with characteristic particles. Particles in cell on right appear to be in invaginations of cell membrane. $\times 32,400$.

PLATE 7

Fig. 10. 96 hr. after infection. Lower magnification of three cells, showing nuclei with marginated chromatin, containing characteristic particles, and many more particles on the surfaces of the cells. $\times 12,000$.

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Quantitative Studies on the Growth of Herpes Virus in HeLa Cells

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SUMMARY: Less than a tenth of the pock-forming particles of the HFEM strain of herpes virus are able to initiate infection in HeLa cells, even after passage in these cells. The pock-forming virus attaches firmly to the cells but most of it remains susceptible to antiserum and presumably does not penetrate the cell. A small proportion of virus initiates infection in the HeLa cells, and, after exposure to more than one HeLa infectious particle/cell, new virus first appears in the cell fraction 12 hr. after infection, and 4 hr. later virus is released into the medium. Virus in the cell fraction can be detected either by disruption of the cells, or simply by removing the cells from the glass with ethylenediaminetetra-acetic acid (EDTA) or saline. This 'EDTA fraction' may contain virus released from the cell surface. An attempt was made to determine the number of virus-yielding cells, by inoculation of whole cells on to the chick chorioallantoic membrane. The proportion which yielded virus was lower than would be expected from the input of HeLa-infectious virus. After removal of superficially attached virus with antiserum, it was not possible to detect infective virus during the latent period in the majority of cells which ultimately released virus.

Herpes virus is well known as a cause of latent infection, and it is our interest in latency which has prompted further investigation of the growth of this virus. Previous work on herpes virus multiplication has been carried out in the chorioallantoic membrane cavity of fertile hens' eggs (Scott, Coriell, Blank & Gray, 1953; Wildy, 1954; Yoshino & Taniguchi, 1956*a, b*) and in explants of rabbit cornea (Scott, Burgoon, Coriell & Blank, 1953). In recent years a more quantitative approach has been possible through the use of cell suspensions and monolayer cultures, and growth studies have been reported by Gostling & Bedson (1956) and Gostling (1956) in chick embryo cells, and more recently by Kaplan (1957) in rabbit kidney cells. We have studied the growth of herpes virus in HeLa cells because of the desirability of propagating cells indefinitely when studying latent infection. HeLa cells are also convenient because they are suitable for isolation of cell clones, and for study of virus release from isolated single cells.

This paper describes some aspects of virus growth in monolayer cultures of HeLa cells. Studies on plaque formation in HeLa cells (Farnham, 1958), cytological observations (Ross & Orlans, 1958), chemical changes (Newton & Stoker, 1958), electron microscope studies (Stoker, Smith & Ross, 1958) and single cell experiments (Wildy, P., Stoker, M. G. P. & Ross, R. W., unpublished) will be given in other communications.

METHODS

Propagation of line of HeLa cells. Human epidermoid carcinoma cells (strain HeLa, Gey) were maintained as stationary cultures on one of the flat sides (3.0×10.0 cm.) of 200 ml. Pyrex babies' feeding bottles. Growth medium consisted of human serum 20 % (v/v), lactalbumin hydrolysate 0.5 % (w/v), and phenol red 0.002 % (w/v), in Gey's (1949) saline, with 100 units penicillin and 100 μ g. streptomycin/ml. For subculture cells were washed three times with calcium- and magnesium-free buffered saline (solution A of Dulbecco & Vogt, 1954) and removed from the glass by treatment with 1/20,000 (w/v) sodium ethylenediaminetetra-acetic acid (EDTA) for 20 min. at 37°. Alternatively, cultures were treated with 1/5000 (w/v) sodium EDTA without previous washing. For virus inoculation continuous sheets of cells were grown in babies' feeding bottles or in 1.7×14 cm. Pyrex test tubes on a rectangular flattened area of 1.0×3.7 cm. Before use the cell sheets were washed with phosphate buffered saline (PBS; Dulbecco & Vogt, 1954) to remove herpes antibody in the growth medium.

Virus experiments were carried out in the following medium: rabbit serum 5 % (v/v), lactalbumin hydrolysate 0.5 % (w/v) and phenol red 0.002 % (w/v) in Gey's (1949) saline, with penicillin 100 units and streptomycin 100 μ g./ml. Reference in the text to medium without further specification, means that this rabbit serum medium was used for experiments.

Cell counts were made in a haemocytometer chamber after pipetting the cells to remove clumps. For rough estimates 100 or more cells were counted in four 1 mm. squares. For enumeration of the proportion of yielding cells eight 1 mm. squares containing a total of 200 or more cells were counted.

Virus strain. The Melbourne line of the HF strain of herpes virus (HFEM strain), which has undergone long adaptation to eggs and mice, was kindly supplied by Dr Peter Wildy as infected egg fluid. The virus was propagated by serial passage in HeLa cells, and seed suspensions were prepared at the eighth or subsequent passage from culture fluids harvested 3–4 days after inoculation with a large dose of virus, when a generalized cytopathic effect was marked. These seed suspensions, which contained between 10^7 and 10^8 pock units/ml., were centrifuged at 700 *g* for 10 min. and dispersed in small volumes, which were stored at -70° without detectable loss of infectivity.

Virus assay. Infectivity was mostly determined by pock counts on the chorioallantoic membrane (CAM) of fertile hens' eggs. With the strain of virus used, this method gave easily countable lesions, and titres were obtained which were 10 or more times the 50 % end-point as determined by tube titrations in HeLa cells, or the titre determined by plaque counts in HeLa cells (Farnham, 1958). For titration the virus suspensions were diluted in distilled water, 10 % horse serum in meat digest broth, or rabbit serum medium, with no appreciable difference in titres obtained.

The chorioallantoic membranes of 12- or 13-day-old white Leghorn chick embryos were inoculated with 0.2 ml. volumes after preparation by the punch method of Nadaje, Tamm & Overman (1955), but the following modification

was introduced. One hour after the inoculation the eggs were tipped into a vertical position with the original air space uppermost, and the holes were sealed with wax. In this way, air enters the original air space and the false air space is obliterated, the chorioallantoic membrane returning to its original position under the shell membrane. This reduces non-specific lesions and spreads the inoculum widely and evenly so that a larger number of pocks can be counted on each membrane. Two days after inoculation the egg was de-embryonated and almost the whole membrane was harvested, washed and spread for counting. Five or more eggs were inoculated with each dilution. The mean count/membrane was determined from groups of four or more eggs with between 5 and 150 pocks/membrane. The mean count on four or five membranes generally had a standard error between 10 % and 20 %. No increase in the number of pocks resulted when the eggs were incubated for 3 instead of 2 days, and the extra day led to overlapping when there were 100 or more pocks/membrane. Eggs from two other breeds of fowl showed the same sensitivity as the white Leghorn breed used.

Dose-response curves showed a linear relationship up to 150 pocks/membrane when counts were made at 2 days (Fig. 1). The absence of serious overlapping of pocks, even with this large number on one membrane, is probably due to the small size of the herpes lesion and wide dispersal of virus by the replacement of the membrane against the shell. Titres obtained by pock count are referred to in terms of 'pock units'.

Though the more sensitive pock technique is satisfactory for investigation of the development of virus during the growth cycle, it was desirable to express virus input in terms of HeLa cell infectivity. At the time this could only be measured as the 50 % end-point and was variable when compared to the egg infectivity. Subsequently, however, Farnham (1958) has developed a reliable technique for counting microscopic plaques in HeLa cell monolayers under fluid medium, and she has found that the plaque-forming titre of the HFEM strain, after thirty-four passages in HeLa cells, is 7.5 % of the pock-forming titre (egg/HeLa ratio = 13). Under certain conditions 60 % of the plaque-forming units (pfu) are adsorbed in 1 hr. and 80 % in 2 hr. from a thin layer of fluid.

From these figures it has been possible, retrospectively, to calculate the approximate virus input in pfu from the titre of the inoculum in pock units. The deduction is subject to the criticism that the ratio and rate of adsorption may vary from one virus preparation to another especially during passage in HeLa cells. Nevertheless, the original egg grown HFEM strain (Pock/HeLa ratio = 7) has a slightly higher relative infectivity for HeLa cells than the same strain passaged 34 times in these cells. It therefore appears that passage in HeLa cells does not markedly change the infectivity of the virus for these cells.

Stability of virus. Because herpes virus is known to lose infectivity rapidly through thermal inactivation, it was necessary to determine its stability in the various media and fluid used, and at the temperatures of the experiments. No significant reduction in infectivity occurred when the virus was stored in medium at -70° for 3 months or at 4° for 1 month. Storage at -20° , however,

reduced the infectivity more than tenfold in 2 weeks. Ten cycles of freezing at -70° and thawing at 37° did not reduce the infectivity when the virus was suspended in medium. Ten cycles of freezing and thawing in distilled water, however, did lower the titre more than tenfold. Exposure to sodium EDTA (1/2000 to 1/20,000) for 2 hr. at 37° did not affect the virus.

The thermal inactivation of the virus is to be the subject of a separate study (Miss A. E. Farnham, unpublished). In medium at 37° the virus begins to lose infectivity after 1 hr., the titre is halved in 6.5 hr., and there is more than a tenfold reduction in 24 hr. During experiments preparations containing virus were kept at 4° until titrated and were only handled at room temperature or 37° for minimal essential periods.

Antisera. Herpes antisera were obtained by selecting specimens from human blood donors; such antisera, when diluted 1/10 in medium had neutralization indices of about 10^3 as determined by pock counts after virus serum interaction for 30 min. at 37° . Alternatively rabbit antisera of about the same strength were prepared by corneal inoculation of rabbits followed by hyperimmunization with infected rabbit brain suspension.

Growth experiments. When repeated cell samples were required, flat-sided tubes containing monolayers of about 10^5 HeLa cells were inoculated with approximately 10^7 pock units (estimated $10^{5.87}$ HeLa pfu) of virus in 1 ml. medium. After 1–2 hr. at 37° the inoculum was discarded and residual extracellular virus was removed by the following standard procedure. The cells were washed three times with 5–10 ml. volumes of PBS, then exposed to 1.5 ml. of herpes immune serum (10 % in medium) for 30 min., washed once with PBS, re-exposed to fresh immune serum for 30 min., washed three times with PBS to remove antiserum and then replaced under medium. At various time intervals thereafter, groups of four tubes were taken for sampling. The medium was removed, the cells washed three times in calcium magnesium free PBS and removed from the glass by exposure to 1/20,000 sodium EDTA. Clumps were broken by pipetting, and the cells were counted. Some of the suspension of intact cells was diluted in medium, with siliconed pipettes, to give suspensions with 10^2 and 10^3 cells/egg, and inoculated on to the chorioallantoic membrane to show the number of pock-forming cells. A sample of the suspension was also centrifuged leaving the cell-free EDTA fraction for virus assay. The remainder of the cell suspension was also diluted and was disrupted by ten cycles of freezing at -70° . The whole of the resulting debris was inoculated into the chorioallantoic membrane for assay of intracellular virus. When repeated samples of cells were not required, growth experiments were carried out in feeding bottles containing monolayers of 2 to 5 million cells. These were generally inoculated with about 10^8 pock units of virus (estimated $10^{6.87}$ HeLa pfu) in 2 ml. medium. After adsorption, washing was carried out as for tubes but with 50 ml. quantities of PBS and 20 ml. quantities of immune serum in medium. The results of the growth experiments are generally expressed in relation to numbers of cells. After exposure to large doses of the HFEM strain of herpes virus, cell counts in fact remained relatively constant for the duration of the experiment.

RESULTS

General characteristics of growth curve

Monolayers of 10^5 cells in a number of tubes were each exposed to $10^{7.87}$ pock units (estimated $10^{6.74}$ HeLa pfu) of virus for 2 hr. and were then washed and treated with immune serum to remove excess virus as described. The medium, EDTA fraction, and cells (disintegrated by freezing and thawing) were sampled from the pooled contents of sets of four tubes at successive time intervals, and assayed for infective virus. Whole cell suspensions taken in the latent period were also assayed to determine the proportion of cells which formed pocks, through release of virus.

In the first experiment cells were disrupted by freezing and thawing in distilled water, a procedure subsequently shown to reduce virus infectivity, so the virus content of medium and EDTA fraction alone are shown in Fig. 2. A second experiment in Fig. 3, with fewer samples, was carried out under the same conditions but with the cells disrupted by freezing and thawing in medium, which does not lower virus infectivity.

These results show first, that following washing and immune serum treatment, some virus still remains in the medium, EDTA fraction and cell debris. It comprises, however, less than 0.005 % of the original inoculum and represents only 1 pock unit/55 cells (Fig. 2) or 1 pock unit/125 cells (Fig. 3). This residual virus in the latent period remains approximately the same or falls slightly until after 9 hr. At 12 hr. there is a marked increase in the virus content of the cell debris and EDTA fraction, but no release of new virus into the medium. The virus content of the medium does not commence to rise until 16 hr. There is thus a delay of 3–6 hr. between appearance of new virus in the cells and release into the medium. The number of pock-forming cells compared to the total is also shown and is unexpectedly low, but this method of estimating the proportion of cells infected will be discussed later in the results.

To prevent loss of virus through re-attachment, and to investigate multiplication after 26 hr., uncomplicated by second cycles of infection, a further experiment was performed in which the cell suspension was diluted in the latent period to prevent re-attachment. The experiment was set up as before with sampling of medium and EDTA fraction, except that 5 hr. after infection, and following the standard washing and immune serum treatment, 6×10^4 cells from each of two tubes were removed with EDTA, diluted in 20 ml. medium, and incubated in a feeding bottle. A control with this number of normal cells diluted and added to a feeding bottle was exposed to virus after 24 hr. and produced no significant fall in pock count after a further 2 hr., indicating that attachment under these conditions was very slow.

The results in Fig. 4 show that the medium from the diluted cells contained more infective virus than the medium from the intact monolayers, suggesting that release from the monolayers is in fact hampered by re-attachment. Furthermore, the concentration of virus from monolayers and the diluted cells was not reduced 23 hr. later, 50 hr. after infection. Since thermal inactivation

would have decreased the infective virus tenfold in this time, and assuming no second cycle of infection in the diluted cells, it must be concluded that release of first-cycle virus continues from 16 hr. into the second day after infection. This might be due to slow continued release from a number of cells or great variation in the length of the latent period for different cells.

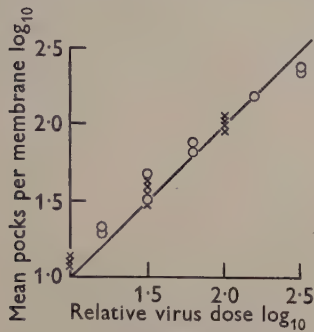


Fig. 1

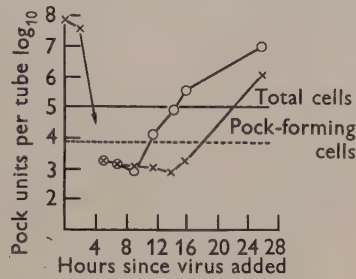


Fig. 2

Fig. 1. Mean pock count per CAM inoculated with increasing quantities of two herpes virus seed suspensions. Seed A = x ; Seed B = o.

Fig. 2. Growth of herpes virus in HeLa cells measured by pock counts. Monolayers exposed to 740 pock units/cell for 2 hr. (equivalent to about 55 HeLa pfu/cell), excess virus then removed by washing and antiserum treatment. 'Total cells' indicate number of cells per monolayer determined by haemocytometer count. 'Pock-forming cells' indicate number of cells per monolayer which formed pocks when inoculated on to the chorioallantoic membrane during the latent period (see text). -o-o-, EDTA fraction; -x-x-x-, medium, including virus in inoculum before and after adsorption.

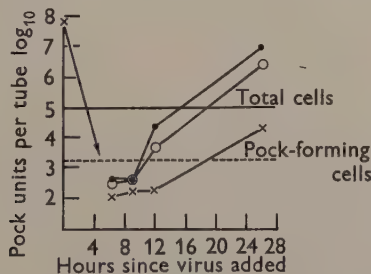


Fig. 3. Growth of herpes virus in HeLa cells measured by pock counts. Monolayers exposed to 740 pock units/cell for 2 hr. (equivalent to about 55 HeLa pfu/cell), excess virus then removed by washing and antiserum treatment. 'Total cells' and 'pock-forming cells' as in Fig. 2. -●-●-, Cell debris after freezing and thawing; -o-o-, EDTA fraction; -x-x-x-, medium (inoculum after adsorption not shown).

An estimate of the amount of virus released/cell is only possible when the proportion of cells yielding virus is known. Only 2.8 % of the cells formed pocks in this experiment, indicating a release of 75 pock units/infected cell at 26 hr. and 144/cell at 50 hr. (not allowing for thermal inactivation). The real proportion of yielding cells may well be higher than that estimated, however (see below), and if so the amount of virus released per infected cell would be correspondingly lower.

Significance of EDTA fraction

Virus appears in the EDTA fraction at the same time as in the fraction released by freezing and thawing. This EDTA fraction contains virus released during the process of detaching the sheet of cells from the glass by the chelating action of the EDTA. The virus released may have been extracellular but released from the cell surface by the EDTA. This would imply accumulation of virus at the cell surface for several hours before release into the medium. On the other hand, it is impossible to exclude damage to a few cells with

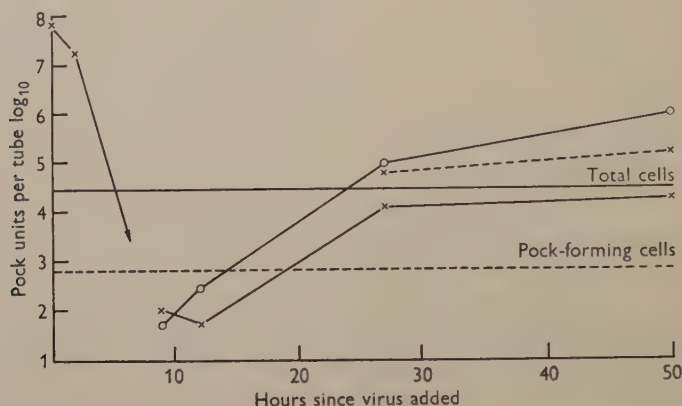


Fig. 4. Growth of herpes virus in HeLa cells measured by pock counts. Monolayers exposed to 740 pock units/cell for 2 hr. (equivalent to about 55 HeLa pfu/cell), excess virus then removed by washing and antiserum treatment. 'Total cells' and 'pock-forming cells' as in Fig. 2. —○—○—, EDTA fraction; —x—x—, medium from original monolayer, including virus in inoculum before and after adsorption; - - x - - x - -, medium from diluted cells.

release of intracellular virus, particularly during the subsequent pipetting. In the experiment shown in Fig. 3 the concentration of virus in the cells at 12 hr. and later was higher than in the EDTA fraction and damage to one-sixth of the cells might release this quantity of virus.

An attempt was made to find whether EDTA had a specific activity in releasing surface virus. Cells infected 26 hr. previously were removed mechanically from the glass with a silicone rubber policeman into warm PBS. The cells were dispersed by gentle pipetting, divided into three portions, and centrifuged. The three pellets of cells were then suspended in PBS, 1/5000 EDTA and 0.5% Difco trypsin, respectively, for 30 min. at 37°. After recentrifugation to remove the cells it was found that more virus had been released into PBS ($10^{7.9}$ pock units/ml.) than into the EDTA solution ($10^{7.35}$ pock units/ml.). Very little virus was present in the trypsin fraction, but the latter even in high dilution caused many deaths amongst the chick embryos, so the titration was unsatisfactory.

This result does not suggest any specific activity of the EDTA but it still remains possible that alteration of the surface of the cell when it comes off the

glass, releases extracellular surface virus. In the latent period in particular, the amount of virus in the cell debris is often lower than that in the EDTA fraction so cell rupture cannot account for the latter.

Virus attachment

The absence at the time of a satisfactory plaque technique in the host cells under study prevented a direct measurement of rate of adsorption, or of multiplicity of infection. Comparative titrations in eggs and HeLa cells had shown that 10 pock units of the HFEM strain of herpes virus grown in HeLa cells were needed to produce a 50 % chance of infection in tube cultures of HeLa cells, and this has been confirmed by the plaque technique developed subsequently by Farnham (1958). Thus the great majority of virus particles are unsuccessful in HeLa cells in the sense that they do not initiate a cycle of multiplication. If the HeLa infective virus alone attaches to the cells, over 90 % of the 'total' virus, i.e. pock units, should remain free and it would be impossible in practice to measure the fall in virus titre over and above loss from thermal inactivation. The following experiment showed that a much higher proportion of the 'total' virus became attached to the cells.

Monolayer cultures of $10^{6.55}$ and $10^{6.7}$ HeLa cells in feeding bottles were exposed to virus at multiplicities of 18 and 0.0013 pock units/cell, respectively. Two ml. inocula were used, giving a depth of about 0.7 mm. of fluid. Samples were removed at 5, 15, 60 and 120 min. for titration by pock counts. The low multiplicity was used in addition to the high dosage because it permitted the virus suspension to be inoculated directly on to the chorioallantoic membrane with only one further dilution step, thus increasing the accuracy of the titration. In addition, virus attachment to an incomplete sheet of HeLa cells was investigated by adding this small inoculum to 10^6 cells spread over the same area of glass in a feeding bottle. As controls to detect thermal inactivation, the same amounts of virus were inoculated into empty bottles and also sampled.

The results are shown in Figs. 5 and 6. From the controls it will be seen that thermal inactivation was insignificant until the second hour. In the presence of the cells, however, about half the virus disappeared from the inoculum in 1 hr., with both low and high dosages of virus and with both complete and incomplete monolayers of cells. Further experiments showed that this was not due to release of virus-inhibitory substances from the freshly washed sheets of cells, or to greater virus inactivation at the slightly lower pH value maintained in the medium on the cells (pH 7.0) as opposed to that on glass (pH 7.2). It is assumed that the virus is attached to the HeLa cells, but as we have seen, most of this pock-forming virus is unable to multiply in these cells.

Residual virus in the latent period

After the standard procedure of washing the infected cell sheets with PBS and two exposures to herpes antiserum, infective virus was always detected during the latent period in the cell fraction and in the medium. This residual virus usually constituted less than 0.01 % of the original inoculum and,

therefore, less than 0.02 % of the 'total' virus which became attached. The virus which was detected in the medium appeared very soon after the antibody was removed. Similar treatment of virus in bottles with no cells left no detectable infectivity so the infectivity of the medium was not due to unneutralized virus detaching from the glass, and must, therefore, have been due to virus coming from the cell fraction. Replacement of medium at hourly intervals in the

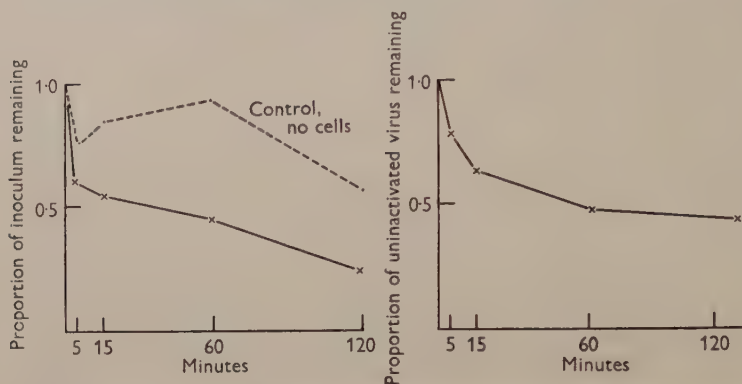


Fig. 5. Loss of pock-forming herpes virus in contact with HeLa cells. Monolayer of $10^{8.56}$ cells exposed to $10^{7.81}$ pock units of virus (18 pock units/cell). Control with no cells shows thermal inactivation. Right-hand figure shows loss of virus after allowing for thermal inactivation.

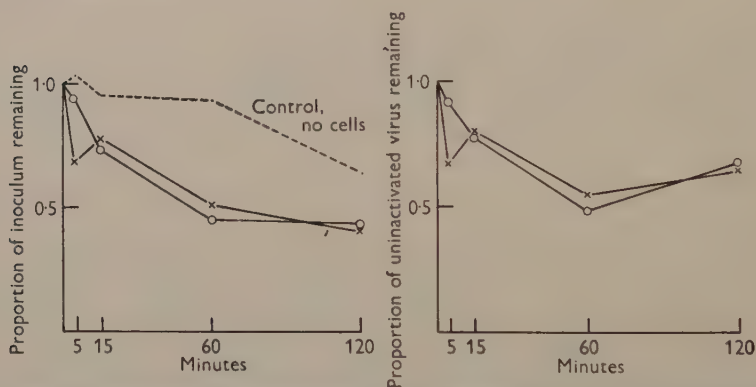


Fig. 6. Loss of pock-forming herpes virus in contact with HeLa cells. Complete sheet of $10^{6.7}$ cells and incomplete sheet of 10^6 cells exposed to 3.81 pock units (0.0013 and 0.0065 pock units/cell respectively). ○-○, 10^6 cells; x-x, $10^{6.7}$ cells.

latent period showed a slightly diminishing rate of release. When the cells were placed in a large volume of medium to decrease the chance of re-attachment, the total virus in the medium was not increased. There is thus no evidence that there is a state of equilibrium between the cell fraction and medium.

The virus associated with the cells usually amounted to less than one infectious particle/10 cells, and it can be found in the EDTA fraction or in the cell debris after disintegration. As already mentioned, it is not possible to

exclude cell damage by EDTA with consequent release of intracellular virus, but in the latent period the cell debris after freezing and thawing ten times contains no more virus than the EDTA fraction. If the virus in the EDTA fraction was originally intracellular it would require disintegration of all the cells to release enough. Microscopic observation of the cells in EDTA solution showed 'bubbling' of an occasional cell but there was certainly no massive breakdown. It seems much more likely that this small amount of virus released by EDTA is at, or on, the surface of the cells, and is released when the cells round up after removal from the glass. The fact that it is not affected by treatment of the cells with herpes antibody does not rule this out when one considers the deep crypts in the HeLa cell surface in which virus may lie (see Stoker *et al.* 1958). Disintegration by freezing and thawing may, in fact, be releasing the same surface virus, because it is not possible to distinguish between virus in an invagination of the surface and true intracellular virus.

Fate of the attached virus

It has been seen that the virus recovered in the cell fraction in the latent period constitutes a very small proportion of that which attaches to the cells. The remainder may have been removed by the washing and immune serum treatment, and some of it may have lost its infectivity as a direct result of its contact with, or penetration of the cells. It was not possible to estimate directly the quantity of virus neutralized by antiserum but the amount removed mechanically by washing could be determined by replacing the immune serum with normal rabbit serum, and titrating the total amount of the virus in the nine changes of fluid. In two experiments 2 million cells were exposed to 52 and 14 pock units/cell, respectively, with similar results. These

Table 1. *Fate of egg-infectious (pock-forming) virus after attachment to HeLa cells. Recovery expressed as % of virus lost from inoculum*

Recovered in wash fluids:		
1st to 3rd	22.0	%
4th to 6th	1.5	
7th to 9th	0.5	
		24
Reappeared in medium after washing		1
Recovered from cells:		
EDTA fraction		4
Cell debris		37
Inactivated during experiment		2
Deficit		32
Total lost from inoculum		100

are summarized in Table 1 in which recovered virus is expressed as a percentage of that lost from the inoculum. It will be seen that almost a quarter of the cell attached virus was recovered in the washing, most of it in the early washes. A control indicated that another small fraction might have been lost by thermal inactivation during the washing period, but there remained 73 % of the attached virus which was not accounted for. Some of this was released after

EDTA treatment of the cells but disintegration of the cells by freezing and thawing revealed a large proportion of this remaining virus in the cell debris. It still appears that there is a fraction of the attached virus which cannot be detected, but the extent or existence of this is difficult to measure because of the lack of accurate determination of the total amount lost from the inoculum.

The bulk of the attached virus is to be found in the cell debris, and it might be assumed that it enters the cells since it is not removed by repeated washing. It has been shown, however, that this virus is almost completely neutralized when the infected cells are exposed to antiserum. In the standard antiserum treatment less than 0.02 % of the adsorbed virus is found in the cell debris compared to 41 % after washing alone. We calculate that nearly all the infective virus found in association with the cells in the latent period is firmly attached at the cell surface, though antiserum-sensitive. The small amount of antiserum-insensitive virus may also be at the surface for reasons already stated, or it may be intracellular.

Estimation of the proportion of cells yielding virus

The object of exposing the cells to large doses of virus was to infect a high proportion of the cells simultaneously and make it easier to interpret changes during virus growth. Exposure to high multiplicity of poek units/cell, however, does not necessarily lead to infection of all the cells. The infectivity of the virus for HeLa cells is at least 10 times less than its poek-forming ability. It was, therefore, necessary to estimate the proportion of yielding cells. This was done by inoculating intact HeLa cells on to the chorioallantoic membrane and assuming that those which were infected would, in due course, release virus which would only form a single poek for each yielding cell. Monolayer cultures infected and washed by the standard treatment were removed from the glass with EDTA and pipetted to form a clump-free suspension. The cells were counted and diluted in medium to give estimated concentrations of 500 and 5000 cells/ml. Two-tenth ml. volumes of cell suspensions with either 100 or 1000 cells, were then inoculated on to the chorioallantoic membrane. The remainder of each suspension was centrifuged and the cell-free supernatant fluids were also inoculated into groups of eggs. The number of poeks formed by the intact cell suspension, less the number in the cell-free supernatant fluid, gave the number of poek-forming cells, which were either carrying superficially attached virus, or alternatively, were yielding virus. The results of various estimations of the proportion of poek-forming cells are shown in Table 2. The input of virus in terms of HeLa pfu/cell has been calculated from the titre of the inoculum in poek units and the time of exposure.

Despite exposure to a very large number of poek units/cell which theoretically should have given a high multiplicity of infection, the growth experiments carried out in flat-sided tubes showed only a low proportion of poek-forming cells (see also Figs. 2-4). The results obtained after infection of monolayers growing in feeding bottles, however, gave somewhat higher number of poek-forming cells in relation to input. This may have been due to the more even distribution of virus in a thin layer over the greater area of the monolayers in

these bottles. The flat side of the test tubes was used and not only was the inoculum deeper but there was a large meniscus, and much of the fluid was in contact with bare glass; moreover, the cells were usually less evenly spread than in the feeding bottles. The proportion of pock-forming cells was not closely related to the input of virus, but the largest estimated multiplicity of infection led to the higher proportion of pock-forming cells. Perhaps a greater range

Table 2. *Proportion of yielding cells*

Monolayers in	Cells exposed to (pock units per cell)	Time of exposure (hr.)	Estimated multiplicity of infection (HeLa pfu) per cell*	Pocks per 100 cells or equivalent volume of cell-free supernate		
				(a) Complete cell suspension	(b) Cell free supernate	(a-b) Pock forming cells
Feeding bottles: residual virus removed	16	1	0.67	4.3	0.02	4.3
by washing and antiserum	37	2	2.2	17.5	1.9	15.6
	43	2	2.5	52.0	6.0	46.0
	79	1	3.3	8.3	0.3	8.0
	200	1	8.4	92.0	4.0	88.0
Feeding bottles: residual virus removed	14	1	0.6	65.4	10.6	54.8
by washing alone	44	1	1.8	21	2.3	18.7
	48	1	2.0	100	18	82
	52	2	3.1	200	100	100
Flat sided test tubes: residual virus removed by washing	65	2	> 3.8	5.8	0.3	5.5
and antiserum	740	2	44	2.0	0.3	1.7
	740	2	44	8.6	1.7	6.9
	740	2	44	3.3	1.0	2.3

* Derived from ratio of pfu in HeLa cells to pock units on CAM (0.075) and proportion of pfu adsorbed to HeLa cells in 1 hr. or 2 hr.

of virus dose might have shown a relationship. Even in feeding bottles, however, the number of pock-forming cells was low considering the size of input, so various factors which might influence the estimation were examined.

A cell which released virus after the latent period might not have time to produce a pock in 2 days. However, leaving the eggs for an extra day before harvesting had no effect on the number of pock-forming cells. This does not exclude the possibility that many cells disintegrate on the membrane before the completion of new virus. Suspending and inoculating the cells at different times during the latent period also had no appreciable effect.

The herpes antibody used in the standard procedure for washing might remain attached to the cells, even after three further washes. This might then neutralize the virus as it left the cell. Increasing the number of washes from three to seven to remove the residual antibody had no effect on the number of pock-forming cells. On the other hand, eliminating the antibody from the washing procedure altogether certainly gave much higher proportions of pock-forming cells (see Table 2). Without antibody, however, it has already been shown that there is a large amount of virus in the cell fraction, which, being antiserum-sensitive, we assume to be superficially adsorbed virus from the inoculum. It is, therefore, probable that most of these cells give rise to

pocks because of contaminating surface virus, and not through releasing newly formed virus. Disintegration of such cells in the latent period by freezing and thawing yields almost the same number of pocks as the intact cells.

It is also possible that with early antiserum treatment the efficiency of infection is low because, after attachment, it takes a long time for the virus on the surface to enter the cell and become antiserum insensitive. Monolayers were, therefore, not exposed to antiserum until 7 hr. instead of the customary 2-3 hr. after infection. This did raise the proportion of pock-forming cells but the EDTA fraction was also increased, so it was difficult to exclude the possibility that the contaminating virus had become antiserum-insensitive though still extracellular, during the extra time, thus increasing the count of pock-forming cells. It is possible that even after antiserum treatment the figures given represent cells carrying contaminating virus alone and bear no relation to the number releasing fresh virus. Contaminating virus, however, should lead to pock formation whether the cell is intact or not, and several preliminary experiments showed that providing they had been treated with antiserum, disintegration of the cells by freezing and thawing produced fewer pocks than intact cells.

Failure to detect infective virus in the latent period

Since disintegration of the cells by freezing in the latent period gave fewer pocks than cells allowed to remain intact, it may be inferred that no intracellular virus was detected during the latent period of those cells which ultimately yield virus. Before this can be taken as evidence for a non-infectious phase in the growth of the virus, however, it is necessary to be sure that the cells, and especially the nuclei, are properly disintegrated and that the method of disintegration, and the resulting cell debris, have no damaging effect on the virus. Various methods of disrupting cells were investigated. Freezing and thawing ten times did not disintegrate the cells fully because the cell count was not decreased. Suspending cells in distilled water showed swelling and bubbling with blurring of the nuclei but no early decrease in count. Exposure of cells suspended in either medium or distilled water to a microblender with stainless steel blades at 20,000 rev./min. for 2 min., however, decreased the cell count by 99 % or more and left very few intact nuclei.

To find out whether normal cell debris, or the method of disintegration, decreased the infectivity of the virus, four batches of 1.8 million cells were mixed with a diluted virus suspension and each was immediately disrupted by one of the four methods described above. Cell debris and virus were inoculated on to the chorioallantoic membrane for assay, and untreated virus suspension was titrated as a control. The result (Table 3) showed no decrease in infectivity of the virus; in fact, the infectivity was slightly increased, though not as much as was found by Kaplan (1957) after similar treatment of herpes virus with rabbit kidney cells.

Disruption of cells in distilled water by the microblender seemed the method of choice and was used in the following attempt to detect intracellular virus

Table 3. *Effect of various methods of cell disintegration and the resulting cell debris on the pock-forming efficiency of herpes virus*

	Pock units per ml. (log ₁₀)
Virus seed—untreated control	3.7
Virus seed mixed with 1.8 million HeLa cells, which were immediately exposed to one of the following methods of cell disintegration before assay:	
Dilution in distilled water	4.0
10 cycles freezing and thawing	3.8
Microblender with cells in medium	3.9
Microblender with cells in distilled water	3.9

in the latent period. A monolayer of cells in a bottle was exposed for 2 hr., to 43 pock units (3.2 HeLa pfu) of virus/cell, washed and treated with immune serum in the standard way, then replaced in medium at 37°. It is estimated that this represents an input equivalent to 2.6 HeLa pfu/cell. Seven hr. after inoculation the medium was sampled and the cells were suspended in EDTA solution, and counted. The cell suspension was diluted in medium to give 500 and 5000 cells/ml., and 0.2 ml. volumes were inoculated into eggs for estimation of the number of pock-forming cells. The remaining cells were centrifuged and the supernatant EDTA fraction removed for assay. The deposited cells were resuspended in distilled water and exposed to a microblender for 2 min. The resulting homogenate was suitably diluted in medium before inoculation directly on to the chorioallantoic membrane. The results of this experiment are given in Table 4. It will be seen that there

Table 4. *Distribution of herpes virus in 3 million HeLa cells during latent period*

	Total pock units (log ₁₀)	Pock units/100 cells
Inoculum	8.11	4300*
Residual virus in latent period		
Medium	5.23	5
EDTA fraction	5.26	6
Disintegrated cell debris	5.11	4
Intact cells (infective centres)	6.14	46

* Estimated adsorption of HeLa infectious virus = 258 pfu/100 cells.

remained in the latent period a small quantity of antiserum-insensitive virus distributed in the medium, the EDTA fraction and cell debris. We have already discussed the possibility that this is all derived from the cell surface. Forty-six out of 100 intact cells gave rise to pocks, but only four pocks could be found/100 cells disintegrated at 7 hr. Thus, some 42 % of cells yielded virus in due course, even though no infective virus could be extracted from them during the latent period.

DISCUSSION

When HeLa cells are exposed to large numbers of egg-infectious particles (pock units)/cell, about half the virus attaches to the cells in 1 hr., but only one pock-forming particle in twenty is able to initiate infection. About a quarter of the attached pock-forming virus can be removed mechanically by washing, but the bulk is firmly attached to the cells, where it can be detected after damaging the cells by freezing. This virus is nearly all neutralized by treating the cells with herpes antiserum so it is assumed to be at the surface. A much smaller quantity of virus, less than one particle/cell, is associated with the cells after antiserum treatment. Since this virus can be released as easily by suspending the cells in EDTA as by freezing and thawing, it is suggested that this is also surface virus, which perhaps lies in the deep invaginations of the HeLa cell membrane and so escapes neutralization. Since most of the 'total' pock-forming virus attaches firmly to the cell surface, the low efficiency of infection is presumably due to failure of the majority to penetrate, possibly due to a limited number of sites on the cell, or alternatively to heterogeneity in the virus population. This low efficiency of the HFEM strain has not been affected by thirty-four passages in HeLa cells.

One of the difficulties in this study has been the lack of a reliable estimate of the number of cells which undergo an infection and release new virus. In the absence of an infective centre count by a sufficiently sensitive plaque technique, the determination of the proportion of pock-forming cells has given variable figures, not closely related to the input of virus. It is possible that this is due to neutralization of the released virus by residual antiserum, but more washes did not increase the proportion of pock-forming cells. Determinations on infected cells which had not been exposed to antiserum gave high counts of pock-forming cells, but this appeared to be due to residual contaminating virus.

Associated studies on the release of herpes virus from single HeLa cells by Drs P. Wildy, M. G. P. Stoker & R. W. Ross (unpublished) indicate that with deduced input multiplicities of between 0.9 and 3.3, 26 % of cells yielded virus, but mostly not until the second day after infection. The proportion and the virus release/cell is not markedly increased when the cells are left free of antiserum, thus confirming that residual antibody is unlikely to be an important factor in decreasing the number of yielders. Direct comparison of the number of yielding single cells and the number of pock-forming cells from the same experiment shows that the estimate based on pock formation errs on the low side. Since leaving the eggs an extra day has no effect, the most likely explanation is that the cells do not all survive on the CAM long enough to make infective virus.

With these limitations in mind, however, and even if we assume the higher figure obtained from single cells, the proportion of those yielding is much lower than that expected from calculation of virus input based on plaque formation. Kaplan (1957), studying the growth of herpes virus in rabbit kidney cells, reported a low estimate of those yielding, judged by plating

infected cells on rabbit kidney monolayers. It is possible that detachment of the cells from the glass, which is necessary for determining the proportion yielding, inhibits or delays virus release.

The evidence that infected cells contain no detectable virus when disintegrated in the latent period is similar to that produced by Wildy (1954), and also requires an estimate of numbers of infected cells. In several experiments it was impossible to detect sufficient virus in the cell debris in the latent period to account for the number of yielding cells especially since the latter is probably an underestimate. Though this suggests that there may be a non-infectious stage in the growth cycle it does not constitute proof unless it can be shown that the pock-forming efficiency of each physical virus particle is high. If the pock-forming efficiency of each particle is low, a few virus particles in a cell in the latent period, even though potentially infectious, would stand less chance of detection than many particles finally released by the cell. Unfortunately this information is not yet available for herpes virus.

Compared to the growth of herpes virus in chick fibroblasts (Gostling & Bedson, 1956) and rabbit kidney cells (Kaplan, 1957), there is a long latent period of at least 9 hr. in HeLa cells before virus appears in the cell fraction (including possibly, the cell surface). Cells infected in the first cycle continue to release virus between 26 and 48 hr. after the original infection, a conclusion which is confirmed by the later studies on single cells. The relatively high rate of thermal inactivation, and the difficulty of knowing the proportion of infected cells makes it impossible to estimate accurately the particles released per cell.

The dynamics of herpes virus multiplication in HeLa cells reported in this paper may be considered with associated studies on the same virus-cell system. Thus, the first microscopic changes in the nucleus appear at 12 hr. after infection, and antigen can be detected in the nucleus by fluorescent antibody staining at 16 hr. (Ross & Orlans, 1958). Chemical determination, however, shows a rise in deoxyribose nucleic acid in the nucleus as early as 6-9 hr. after infection, well before the appearance of new infective virus (Newton & Stoker, 1958). It is not yet known whether this nucleic acid is a precursor of the virus or whether it is an inessential by-product of virus growth.

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Ammonium Alginate Wool as a Filter for Collecting Micro-organisms from Large Volumes of Air

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SUMMARY: Ammonium alginate wool has proved to be a suitable collecting filter for estimating a small number of micro-organisms in a large volume of air.

A large supply of air free from micro-organisms is needed for certain modern commercial processes, such as submerged fermentations, which depend upon aeration and the control of infection. Consequently, it is necessary to test this air for sterility. Most methods of estimation are unsuited to lengthy tests and to large quantities of air, for instance the membrane filter (Haas, 1956), which, though efficient, offers a high resistance to airflow. Sodium alginate wool was found by Richards (1955) to be a suitable filtering material, and, as it is water-soluble, it can be dissolved for plating out; it cannot be purchased, but it is readily formed when calcium alginate is added to a solution of certain sodium salts (Higgins, 1950). Ammonium alginate is available, and is, if anything, slightly better for the purpose than the sodium salt; its ability to collect the micro-organisms from air has been tested by passing artificially infected air afterwards through a membrane filter, which showed that the alginate had collected all the organisms of the size of yeasts or mould spores, and more than 99 % of the spores of *Bacillus subtilis*.

METHODS

Description of apparatus. The sampler designed by Richards (1955) was made of metal tubing and had cross-wires to hold the column of wool in place. The writer has used a Quickfit and Quartz B 19 cone, the wool being held by a constriction, which was blown against a carbon rod to give an internal diameter of $\frac{5}{16}$ in. (Fig. 1).

One gramme of ammonium alginate 50 denier/12 filament, garnetted waste, from Courtaulds Ltd., Cross Street, Manchester, was divided into five parts, each of which was shaped into a disk to fit into the tube, the five disks forming the filtering column. The tube was plugged at each end with non-absorbent cotton-wool and placed in a glass container, which was also plugged. The whole was autoclaved for 30 min. at 10 lb./sq.in. An alternative method of sterilizing, in a dry oven at 110° for 12 hr., was abandoned because it made the alginate wool brown and brittle. Air containing the test organism, either as an aerosol or as a dust, was blown through the apparatus at speeds varying from 1 to 5 cu.ft./min., and then through a membrane filter (Oxoid) to trap any organisms which had passed through the alginate. DuBuy, Hollaender &

Lackey (1945) gave an excellent description of sampling devices and their efficiencies; they recommended the atomizer bubbler, but the present writer found that it offered too large a resistance to airflow, and the membrane filter was considered more suitable.

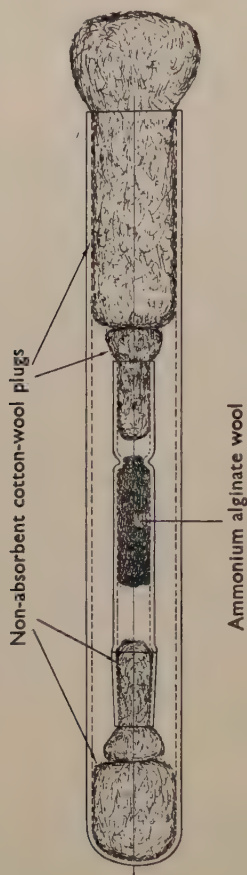


Fig. 1

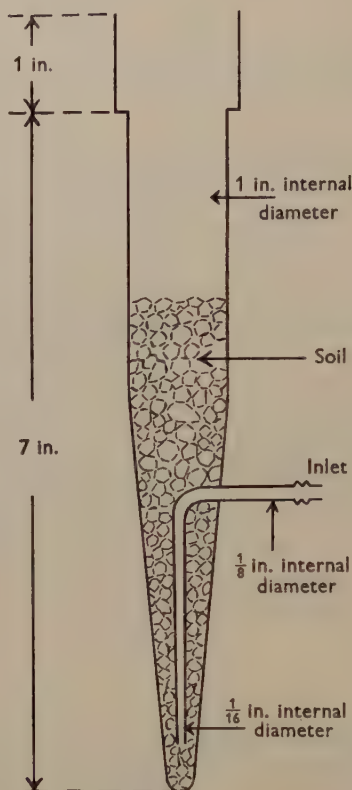


Fig. 2

Preparation of aerosol. The aerosol of the test organism was prepared by passing cold dried air through a small glass atomizer containing a heavy suspension of spores of *Bacillus subtilis*. The relative humidity and temperature of the effluent air were recorded.

Preparation of dust. Soil that had passed through a 20-mesh sieve, but had been retained by a 30-mesh sieve, was sterilized, inoculated with an aqueous suspension of spores of *Bacillus subtilis*, dried in a vacuum desiccator, and placed in the apparatus shown in Fig. 2. The velocity of air at various humidities was adjusted by means of a rotameter to cause a continuous agitation of the soil.

Plating out. By means of a sterile glass rod, the alginate wool was pushed from the tube into 40 ml. of sterile 0.5 % (w/v) dipotassium hydrogen phos-

phate solution. (This buffer was needed because 1 g. ammonium alginate dissolved in water and mixed with nutrient agar decreased the pH of the agar from 6·8 to 4·6; on such a plate the growth of *Bacillus subtilis* was greatly retarded, though *Saccharomyces cerevisiae* and a *Penicillium* sp. were not affected.) Samples of the phosphate solution were mixed with 20 ml. quantities of glucose nutrient agar at 45° and were plated out and incubated at 30° for 3 days. The Oxoid membrane was placed in 20 ml. of 0·5 % (w/v) NaCl solution in a flask with some beads and mechanically shaken for 10–15 min.; samples were then mixed with nutrient agar and plated out.

RESULTS

Table 1 gives the results for aerosol tests, three out of four of which showed 100 % retention of spores of *Bacillus subtilis* by ammonium alginate wool. Table 2 gives five results for dry dusts which are representative of 27 tests carried out; all individual tests gave efficiencies greater than 99 %. The alginate wool must be evenly packed, for a deliberately badly packed tube gave an efficiency of only 97·75 %.

Table 1. *Aerosol tests with spores of Bacillus subtilis*

Filter	Total count	Relative humidity	Temperature (° C.)	Efficiency (%)
Alginate wool	400,000	60	29	99·99
Oxoid membrane	8			
Alginate wool	240,000	60	29	100
Oxoid membrane	0			
Alginate wool	186,000	60	29	100
Oxoid membrane	0			
Alginate wool	180,000	60	29	100
Oxoid membrane	0			

Table 2. *Spores of Bacillus subtilis as a dry dust*

Filter	Total count	Relative humidity	Temperature (° C.)	Efficiency (%)
Alginate wool	240,000	50	21	99·9
Oxoid membrane	240			
Alginate wool	400,000	60	19·5	99·13
Oxoid membrane	3,500			
Alginate wool	280,000	70	20·5	99·46
Oxoid membrane	1,500			
Alginate wool	140,000	80	21	99·79
Oxoid membrane	300			
Alginate wool	400,000	90	22	99·94
Oxoid membrane	260			

Depth of penetration of the alginate wool column by dry spores of Bacillus subtilis. Five wads of ammonium alginate wool (each 0·2 g.) were placed adjacent to one another in a tube; air containing dry spores was passed, and

each of the wads plated out. The results show that the penetration of single spores is probably logarithmic, as found by Humphrey & Gaden (1955) in the case of a fibrous filter.

CONCLUSIONS

Ammonium alginate wool used as an air sampler is 100 % efficient for organisms of diameter greater than $2\ \mu$, which includes the majority of yeasts and moulds. It varied in efficiency from 99.13 to 99.96 % with dry spores of *Bacillus subtilis* in concentrations ranging from 5000 to 500,000/cu.ft. air.

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A Comparative Study of Cytoplasmic Inclusions (Volutin Granules) in Different Species of Trypanosomes

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SUMMARY: The literature of 'volutin' granules is reviewed in an attempt to clarify the meaning of the term in respect to trypanosomes. Fifteen species of trypanosomes have been examined by phase-contrast microscopy and the morphology and natural history of their cytoplasmic inclusions described. It is suggested that the formation of inclusions in trypanosomes circulating in the blood of mammals represents a constant phenomenon which is closely related in time to the immune reaction of the host; other evidence suggests that this relationship is not fortuitous. In addition, the formation of inclusions in *Trypanosoma rhodesiense* and *T. lewisi* appears to be related to polymorphism, notwithstanding the wide differences in the manner and time in which polymorphism (and associated cytoplasmic inclusions) occur within their cycles in the vertebrate host.

This paper describes the morphology and discusses the significance of inclusions which occur in the cytoplasm of trypanosomes circulating in the blood of vertebrates. My attention was first drawn to these inclusions by their presence in many laboratory strains of trypanosomes, by their complete absence from others, and by the resemblance of these inclusions to those produced by Antrycide and other drugs in strains in which these inclusions do not normally occur. Because of this resemblance I suggested (Ormerod, 1951) that these inclusions which occur naturally might be produced—in the same way as by drugs—by basic or acidic substances, in this instance derived from the host, circulating in the blood and able to be absorbed by the trypanosome and to disrupt the ribonucleoprotein in the cytoplasm into its constituent protein and ribonucleic acid. I began this study of the morphology of these inclusions to obtain evidence for or against this hypothesis. Similar inclusions occur in the leptomonad and crithidial forms of trypanosomes, both in culture and in the gut of invertebrates; they also occur in flagellates and other protozoa generally that are not adapted to vertebrate blood. These will be considered only in relation to the main phenomenon, namely, the presence or absence of inclusions in blood trypanosomes; it is hoped to consider them in more detail in a later paper.

Historical survey

Volutin. The term 'Volutans granules' was coined by Grimme (1902) to denote granules which occur in various plant, fungal and bacterial cells with staining properties resembling those of *Spirillum volutans*; Meyer (1904) subsequently altered the term to 'volutin' because he realized that it represented a chemical substance or reaction type that was common to most, if not all,

forms of life. He devised a series of tests based on the stability of the granules to water, acids and alkalis, their staining properties with methylene blue and its retention under the action of solvents. Meyer's insight into the nature of volutin was shown by his comparison of volutin granules present in yeast with the substance that had been isolated from yeast by Kossel (1882) and called 'Hefe-nukleinsäure'; Meyer showed that there was a close similarity between the reactions of these substances and believed that they were in fact the same. Since that date the chemistry of nucleic acid has developed enormously and Kossel's yeast nucleic acid, now called 'ribonucleic acid' is recognized as being present in the nucleus and cytoplasm of all living cells; the nature of the granules has, however, been obscured, partly because of the use of the term 'volutin' that has satisfied workers who, having performed Meyer's tests, were content with this identification and failed to inquire further about their nature, and because of controversy (Guilliermond, 1910) about whether these granules should be called 'volutin' or 'metachromatic'. The term 'metachromatic granules' certainly seems to have been the first used to describe these structures (Babes, 1895) but has the disadvantage of making the phenomenon of metachromasia the main feature of identification. This is unsatisfactory because: (a) as a chemical phenomenon it is imperfectly understood, being related more to the concentration of the dye than to the nature of the granule (Michaelis, 1944); (b) it is known to give positive results with substances of a totally different nature such as heparin, dextran sulphate (Walton, 1952), metaphosphate polymers (Dämle & Krishnan, 1954) and mucopolysaccharides (Lison, 1935); (c) as will be shown later in this paper, inclusions apparently of the same nature sometimes do not stain at all.

The phenomenon of metachromasia has, however, directed attention to a constituent of the volutin granules in yeast, and it has been demonstrated (Wiame, 1947) that a substance can be extracted from yeast and that its presence or absence determines the metachromasia of the volutin granules. This substance has been identified (Ebel, 1952) as 'Graham's salt', an inorganic long-chain metaphosphate polymer having the property of combining with proteins and with metallic ions. It is uncertain, however, whether these findings are of significance to the study of cytoplasmic inclusions in trypanosomes. In protozoological literature the interest in volutin stems entirely from Meyer's work, but after the description of volutin granules in various protozoa (in *Coccidia*, Kunze, 1907; in trypanosomes, Swellengrebel, 1908; in *Sarcosporidia*, Erdmann, 1910; in *Haemogregarines*, Reichenow, 1910) protozoologists appear to have lost interest in this barren concept.

Swellengrebel (1908) first gave an adequate description of volutin granules in trypanosomes and identified their substance by Meyer's tests. His description is sometimes difficult to follow since it involves the extrusion of these granules from the nucleus along an 'achromatic axial filament'. It is evident from his figures that he was dealing with inclusions similar to those being considered here, and I believe that his description represents the observation (which will be referred to in the section on Results) that the inclusions in *Trypanosoma rhodesiense* are often to be found strung like beads

in one or two chains in the long axis of the trypanosome anterior to the nucleus. These granules do not always take up the stain, and under these circumstances give the appearance of an achromatic filament. Similar observations were also made by Salvin-Moore & Breinl (1908). They noted granules which stained with a methylene-blue + safranin stain and which were present in all trypanosomes at some times, absent at others, and had no connexion with the nucleus.

A single granule was described in the mature blood forms of *Trypanosoma lewisi* by Wenyon (1926); this granule occurred near the nucleus and stained red with Giemsa. A similar granule was described by Wolcott (1952) as taking part in nuclear division in the early forms of *T. lewisi*. I have seen instances of this granule; it can often be seen in occasional trypanosomes in a heavy infection of *T. lewisi*, but I am unable to say whether it is in any way related to the inclusions described in the present paper. Another granule was described by Minchin (1909), in osmic acid-fixed specimens of *T. lewisi*; this finding will be referred to in the section on Results.

The most important study hitherto of volutin granules in trypanosomes was made by van den Berghe (1942). He returned to the view of Meyer (1904) which had again been made acceptable by the work of Caspersen & Brand (1941) who studied the ultraviolet absorption of yeast volutin and demonstrated its probable identity with ribonucleic acid. Van den Berghe used the technique of Brachet (1941) of identification by specific digestion of the granules with pancreatic ribonuclease; although his results must be considered with caution both because of difficulties of interpretation (Ormerod, 1951) and because the enzyme available at that period was contaminated with protease, there is little doubt of the general validity of his conclusions that volutin granules in trypanosomes consist largely of ribonucleic acid, that they are not disintegrated products from the nucleus and that they have no direct connexion with the nucleus or with nuclear chromatin.

In cold-blooded vertebrate hosts cytoplasmic granules appear to be a frequent feature of trypanosomes and have been described in *Trypanosoma diemyctyli* in the American triton by Nigrelli (1929) and by Barrow (1954). Barrow studied the granules by histochemical methods and realized their acid nature but, adopting the interpretation of Lison (1935), he took their metachromatic staining to indicate the sulphate groups of a mucopolysaccharide. Gerzeli (1955), studying *T. granulosum* in the eel (*Anguilla vulgaris*), *T. remaki* in the pike (*Esox lucius*) and *T. wenyoni* in the bullhead (*Gobius fluvialis*), used the more specific 'periodic acid-Schiff' reaction (Hotchkiss, 1948), and was unable to detect polysaccharide; he was, however, able to identify ribonucleic acid in the granules of these trypanosomes and in the granules present in laboratory strains of *T. brucei* in mice and *T. gambiense* in guinea-pigs.

In *Toxoplasma gondii* granules occur which have been observed under phase contrast (Lelong & Desmonts, 1951) and by the electron microscope (Bringmann & Holz, 1953).

Vital staining with granule formation occurs in *Toxoplasma* with a solution of methylene blue (Sabin & Feldman, 1948), but after treatment with an

immune serum approximately nine-tenths of the organisms fail to take up the stain. Lelong & Desmonts were able to show that this was because granule formation had already taken place under the action of the serum and had inhibited subsequent staining, the change being visible under phase contrast within a minute of applying the serum; and Bringmann & Holz identified the granules as being composed largely of ribonucleic acid, but regarded their formation as unspecific since they were produced also by antibiotics (unspecified).

As stated above, the first intention of the present work was to explore the hypothesis that the granules in the cytoplasm of untreated trypanosomes might be produced by some circulating substance derived from the host and absorbed into the trypanosome. The second purpose of this work was to establish whether these inclusions were of practical significance or not, that is to say, whether they occurred in naturally-transmitted infections and in infections occurring in the field, particularly those of medical and economic importance. It would have been of interest but of little practical value had they been found to be laboratory artifacts. For this purpose the natural history of the granules, that is to say, the morphology, the times of appearance and disappearance, their relationship to changes in the trypanosome, to the vertebrate host, and to the means of transmission (by insect vector or by syringe) has been studied. The study has been made in as many species of trypanosomes as were available, in the belief that this phenomenon in circulating trypanosomes might be in association with the adaptation of the flagellate to a parasitic existence in vertebrate blood, and that it might be expected to vary at different phases of evolution throughout the genus *Trypanosoma*.

METHODS

As far as possible natural infections have been used in this study. It has been known since the work of Bruce *et al.* (1915) that strains of African pathogenic trypanosomes can change considerably in their morphology and behaviour when isolated in the laboratory. Where natural infections or infections transmitted in the laboratory by arthropod vector could not be studied, strains of recent isolation were used. It is from these three types of strain that the main conclusions of the work are drawn, while strains which had been passaged artificially in the laboratory for long periods were used only for comparison to show the range of adaptation to which the different species were capable in respect of formation of inclusions.

Observations were made with the phase-contrast microscope (Cooke, Troughton & Sims; $\times 95$ oil-immersion objective). For this purpose a small drop of blood was placed on a coverslip which was applied to a slide faced with a gel of 1 % (w/v) agar in physiological saline. The blood spreads by capillary action between the agar and coverslip to form a single layer of cells, the corpuscles being arranged edge to edge to form a 'phalanx'. Trypanosomes are best observed either at the edge of the phalanx, or if the coverslip is moved gently from side to side, in gaps which appear in the phalanx; trypanosomes

moving in the unbroken phalanx cannot accurately be observed since the degree of contrast of inclusions against cytoplasm is diminished by the proximity of the more refractile red blood corpuscles. By this method, for which I am indebted to Mr J. Smiles, it is possible to observe living trypanosomes under oil immersion as clearly as if stained on a dry smear, and with the cytoplasmic inclusions displayed with greater clarity than by any other method. Photography is, however, rarely possible because the movement is still rapid, still (to some extent) three-dimensional and because of the low transmission of light by the phase-contrast system. All illustrations used in this paper are therefore free-hand drawings.

Observations were also made on dry fixed preparations stained with 'Revector' Giemsa stain. In spite of the convenience of obtaining permanent preparations it was found in practice that this was an unreliable method for detecting the presence of inclusions since (as will be shown later) several species of trypanosomes appeared capable of undergoing a change whereby the inclusions were no longer able to take up the stain.

Histochemical methods were used at first in an attempt to study the chemical nature of the granules; some of these methods have been described and their disadvantages given in relation to this type of work (Ormerod, 1951). In this former paper the main reliance for the identification of nucleic acid was the tetrazonium reaction of Mitchell (1942) and Danielli (1947). Barnard & Danielli (1956) have since shown that this reaction is not specific for purines and pyrimidines as was then supposed, but reacts specifically with histidine; although it is usually positive in deposits of nucleic acids, it is in fact an indication of the presence of protein and not of nucleic acid. For this reason, because of the unreliability of other techniques available, and because so many previous workers have been led to wrong conclusions by the use of histochemical technique, I felt that it was necessary to confine these observations to morphology and reserve for a later date a more precise study of the chemical nature of granules isolated from suitable species of trypanosomes.

RESULTS

The species studied are arranged in a classification adapted from Hoare (1957) and from Wenyon (1926).

Group I

(1) *Trypanosoma lewisi* (Kent, 1880). Three strains of *T. lewisi* were examined.

Strain 1, the 'Winches' strain, was isolated 3½ years previously from a wild *Rattus norvegicus* at Winches Farm, St Albans, Hertfordshire. The strain was passaged by syringe in laboratory rats and produced high blood infection which would last in some rats for long periods (maximum 12 months) after which it would vanish from the blood. The monomorphic adult form, Pl. 1, fig. 1, that is to say, the form circulating after 15 days of passage—contained a single granule in the region of the kinetoplast (two inclusions were sometimes present in these forms). At the beginning of the infection from the 6th to

12th day when the infection was polymorphic, immature forms which were broader and thicker than the adults were present; these contained multiple inclusions (Pl. 1, fig. 2) which were faint and could not be counted with ease as they were grouped together in the region of the kinetoplast. They were most obvious at about the 10th day after which the number of trypanosomes so affected declined until about the 15th day, when all trypanosomes remaining in the circulation were of the adult form. The decline in the granular trypanosomes coincided with the overall decline in total numbers.

In dry smears stained with Giemsa these inclusions did not stain and could only sometimes be seen as gaps in the cytoplasm. In trypanosomes with multiple inclusions it was easy, by comparing stained and living organisms, to show that the inclusions were distinct from the kinetoplast. This was not so easy in the adult trypanosome where one inclusion only was present. I believe, however, that they are distinct and that the inclusion usually lies posterior to the kinetoplast, as did the osmiophilic granule noted by Minchin (1909).

Occasional single inclusions (c. 1/100 trypanosomes) were seen in dry fixed films stained with Giemsa; these stained lightly and were eosinophilic, did not appear under phase contrast, and may correspond to those referred to by Wenyon (1926) and by Wolcott (1952). Passage through the flea *Xenopsylla cheopis* did not change the behaviour or appearance of this strain.

Strain 2. 'Rn 86' was observed in wild *Rattus norvegicus* collected three and a half years later and within 200 yards of the site of collection of the 'Winches' strain. In the original rat approximately one in ten trypanosomes contained two to three inclusions and there was slight polymorphism. Passage was obtained in laboratory rats. At the third passage 75 days after isolation, the strain was examined (parallel observations were made with Strain 1 at the same time); the appearances in the two strains were identical but maximal inclusion formation occurred 4 days later in strain 2. Disappearance of inclusions and assumption of the adult form was similarly delayed.

Nineteen months later strains 1 and 2 were indistinguishable, the time of maximal inclusion formation varied from 5 to 15 days depending on the age and in particular on the strain of rat used for passage; there was no variation, however, in the appearance of the trypanosome, or of the inclusions.

Strain 3 was observed in a wild *Rattus rattus* trapped on a ship that had arrived from Jamaica. The rat had been dead for some hours but the trypanosomes were motile and of the adult type, resembling those in Pl. 1, fig. 1. Passage into laboratory rats failed.

(2) *Trypanosoma duttoni* Thiroux 1900. A single strain of this organism was examined in a wild house-mouse *Mus musculus*. Five individuals were seen under phase contrast, four were adult forms, and contained a single inclusion in the region of the kinetoplast. One was broader than the others (presumably an immature form) and contained inclusions (number not noted) between the region of the kinetoplast and the nucleus. Broad forms were present in the stained film and these contained inclusions that stained violet. Passage into laboratory mice failed.

(3) *Trypanosoma grosi* Laveran & Pettit, 1909. Two strains of this trypanosome (Pl. 1, fig. 3) were examined in their original host, the long-tailed field mouse *Apodemus sylvaticus* trapped in the woods and hedgerows at Winches Farm. They were passaged into animals of the same species which, having been trapped and kept in captivity for several weeks without showing infection, were presumed clean.

Table 1. *Trypanosoma grosi* infection at first syringe passage into wild caught long-tailed field mice (*Apodemus sylvaticus*). Table showing the relative numbers of inclusions seen under phase-contrast and in Giemsa-stained preparations

	Day	By phase-contrast			By Giemsa-staining		
		Trypanosomes		Inclusions: no./trypano- some	Trypanosomes		Inclusions: no./trypano- some
		No. seen	No. with inclusions		No. seen	No. with inclusions	
Mouse 1	4	2	0	0	3	3	2, 2, 3
	5	—	—	—	4	1	3
	7	29	all	2-10	—	—	—
	14	—	—	—	7	2	7, 2
	21	10	1	2	5	2	1, 7
	38	No trypanosomes seen					
Mouse 2	7	20	'Many broad forms with inclusions'	—	—	—	—
	14	—	—	—	40	10	1-6
	21	20	Many	—	20	5	2, 3, 2, 3, 2
	24	Animal died		—	—	—	—

Strain 1. On capture twenty-one trypanosomes were seen under phase contrast; one was broad and contained five inclusions. In a dry film on the same day stained with Giemsa, out of forty organisms, seven contained from two to five inclusions. The strain was passaged by injection into two *Apodemus sylvaticus* and the results of this infection are shown in Table 1. The inclusions were in all cases near the region of the kinetoplast but not so closely confined to this region as in *Trypanosoma lewisi*. They were chiefly in the broad forms and in these broad forms were present in their clearest form and greatest number. Although they were similar to those seen with phase contrast in *T. lewisi* they were slightly more refractile and more easily seen, in addition they were frequently (perhaps always) stainable with Giemsa. The observations in Table 1 show in addition that inclusions (and the broad forms in which they occur) were present—in contrast to *T. lewisi*—throughout much of the duration of the infection.

Strain 2 showed an infection with narrow forms only, without inclusions; this strain was not passaged.

(4) *Trypanosoma evotamys* Hadwen, 1912. This trypanosome was seen on three occasions in the bank vole *Clethrionomys (Evotamys) glareolus*; in each instance only the typical *lewisi*-like adult was observed and passage was not obtained.

(5) *Trypanosoma acomys* Wenyon, 1909. A spiny mouse *Acomys* sp. was kindly sent by Dr A. Zuckerman from Israel. Faint inclusions with little refractile power were present in most forms, but little idea of the significance of these inclusions was obtained before the infection died out.

(6) *Trypanosoma cruzi* Chagas, 1909. Strain 1. 'Sonya' strain (Garnham, 1956) was studied in newly weaned rats and in mice. These were infected with the dejecta of laboratory-bred *Triatoma infestans* which had been fed on a human case of Chagas' disease. The appearances of the organism (Pl. 1, fig. 4) were constant from the time the animal showed the infection (about 12 days) to its death. The inclusions were large, constant in size, and distributed on either side of the nucleus; too closely packed to be counted in a motile organism, they did not stain with Giemsa.

Strain 2. The 'Y' strain had been isolated in 1950 and passaged in rats and mice by syringe and cyclically by *T. infestans* (Rego & Garnham, 1956). The appearance was identical with strain 1.

Strain 3. 738 LM strain (Liverpool School of Tropical Medicine). Passaged by syringe in mice for a number of years; the appearances were identical with those of strains 1 and 2.

(7) *Trypanosoma nabiasi* Railliet, 1895. This was not seen under phase contrast. Slides stained with Giemsa kindly lent by Dr M. Singh Grewal showed basophilic inclusions in the broad immature forms similar to those described above for *T. grosi*.

(8) *Trypanosoma avium* Danilewsky, 1885. I was able to examine this trypanosome from the blood of a jackdaw (*Corvus monedula spermologus*) infected by eating numerous louse flies (*Ornithomyia avicularis*) which had been obtained from rooks (*C. frugilegus frugilegus*) (Baker, 1955). Three specimens of this very occult organism were seen under phase contrast; none contained inclusions. There was no evidence of inclusions in stained preparations.

Group II

(9) *Trypanosoma vivax* Ziemann, 1905. Strain 1. 'Vom' strain: Desowitz & Watson (1953). This strain was adapted to syringe passage in rats which it killed within 4 days, such was the virulence of its infection. The cytoplasm was clear throughout and in repeated examination no inclusion was ever seen.

Strain 2. Examinations were made in the field at Kenaba, Gambia. Two zebu bulls from a tsetse-free area north of Kaolack, Senegal, were shipped over the Gambia River (Barra to Bathurst). On day 1 the blood contained no trypanosomes, neither did the blood of the other forty-eight cattle in the same herd. The blood remained negative until day 6 when one animal showed a blood infection with *Treponema theileri* (this is probably of no significance but has been recorded because of possible immune relationships between the two types of infection (Thompson & DeMuro, 1932). On day 7 the bulls were carried by lorry to Tankolar (a village on the south bank of the Gambia River, and the north shore of the Bintang promontory) which was heavily infested with *Glossina morsitans*. On the 14th day both bulls showed an infection with *Trypanosoma vivax* which remained constant, at the rate of about 1 trypano-

some/50 microscopic fields ($\times 600$ magnification), until the death of the animals on days 19 and 27, respectively. It is uncertain when the infective tsetse bites occurred, but the bulls were twice exposed to tsetse (*a*) on the 4- to 5-day journey from Kaolack, i.e. in the 7 days preceding day 1, giving an incubation period of 14–21 days, or (*b*) at Tankolar from day 7 onwards, giving a maximum incubation period of 7 days. The longer period is the more likely, both on general grounds and from the low-grade infection which followed. The appearance of the trypanosomes was constant in both animals throughout the infection: all trypanosomes contained inclusions which lay on either side of the region of the nucleus. There were also inclusions in the region of the kinetoplast but not invariably in this position; from one to five inclusions/trypanosome (seldom more than three) were seen. The inclusions did not stain with Giemsa.

Group III (*Congolense* group)

(10) *Trypanosoma congolense* Broden, 1904. I have examined no strain of *T. congolense* that has been transmitted other than by syringe. Numerous laboratory strains which I have examined show wide variation. Acute infections are sometimes without inclusions, more chronic infections show fine granules; inclusions or sometimes refractile bodies the size of (but in the wrong position for) the nucleus. I have not yet seen stained cytoplasmic inclusions in this species but I am aware that others have.

(11) *Trypanosoma simiae* Bruce *et al.* 1911. I examined one strain of this trypanosome at Vom. It had been isolated from a domestic pig which had been bitten by wild *Glossina morsitans*. The strain had been passaged twice by syringe in rabbits and finally by fly into a pig—which developed a heavy infection within 6 days. The cytoplasm of the trypanosomes was clear and without inclusions. The pig died on the following day.

Group IV (*Brucei* group)

(12) *Trypanosoma brucei* Plimmer & Bradford, 1899. I examined only syringe-passaged strains of this species. All strains had become more or less monomorphic on passage, with irregular and scanty inclusions. Acute strains killing the animal in 3 or 4 days were free from inclusions.

(13) *Trypanosoma rhodesiense* Stephens & Fantham, 1910. *Strain 1*. The 'Maun' strain had been isolated from man in Bechuanaland by Dr Hansford. The fly involved in this area is *Glossina morsitans*. A guinea-pig was inoculated from the original patient and I examined the blood 10 days later. An infection of the order of 2 trypanosomes/field ($\times 600$ magnification) was established. The infection consisted of approximately equal numbers of long thin and of short stumpy forms. The long thin forms had clear highly refractile cytoplasm, and the short stumpy forms contained inclusions; forms intermediate in length were also present and these were intermediate also in the number and size of the inclusions that they contained. As the infection declined, the short stumpy forms became relatively more numerous and the granules that they contained became more numerous and larger, until on day 17 of the infection

the few remaining trypanosomes consisted of short, stumpy, posterior nuclear forms with many large granules, some as large as nuclei but spherical and more highly refractile. On day 18 the infection was absent, but slowly re-established itself to *c.* 10 trypanosomes/ $\times 600$ field at day 40; it remained at about this value with little fluctuation in the numbers of long and short forms (the granules remaining confined to the short forms) until the death of the animal on day 92.

The infection in rats was similar; since larger numbers of trypanosomes circulated, it was easier to study. Pl. 2, fig. 5 shows organisms of the Maun strain, 11 months after isolation, differing in no material way either in appearance or behaviour from when it was first isolated. All inclusions are large

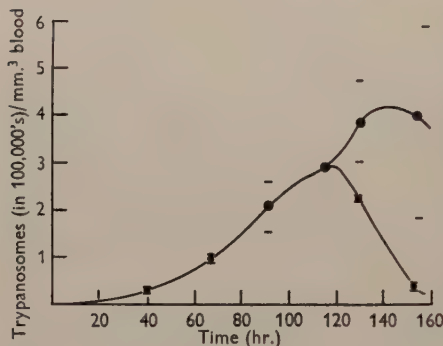


Fig. 1

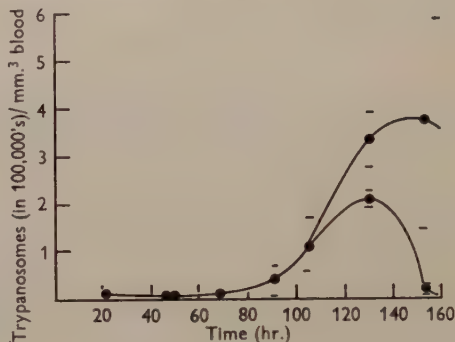


Fig. 2

Fig. 1. *Trypanosoma rhodesiense*. 'Maun' strain. Graph showing the mean total number of trypanosomes (with standard deviation) plotted against time. The upper limb of the bifurcation shows the course of the infection in two rats (out of the total six) in which the crisis was delayed.

Fig. 2. *Trypanosoma rhodesiense*. 'Maun' strain. To be read in conjunction with Fig. 1. Graph showing the mean number of trypanosomes containing inclusions. Until 60 hr. the trypanosomes were almost without inclusions, the proportion then increased until the crisis, after which all trypanosomes in the circulation contained inclusions.

and easily counted; absent in long, thin forms, present but scanty in intermediate forms, and very well marked in short stumpy forms, in particular posterior nuclear forms. Fig. 1 shows the growth of the infection up to the time of crisis; in two out of the six rats used in these observations, the crisis was delayed and the course of their crisis is shown in the upper bifurcation of the curve. Figure 2 shows the increase after 60 hr. of trypanosomes containing inclusions. Figure 3 shows the degree to which trypanosomes circulating in the blood were affected by inclusions. Up to 40 hr. a relatively large number of trypanosomes contained inclusions and these were most probably trypanosomes which had contained inclusions before injection; between 40 and 60 hr. trypanosomes containing inclusions were very scanty, but from 60 hr. onwards the proportion containing inclusions, and from 120 hr. the number of inclusions in each affected trypanosome, increased until the crisis when nearly all circulating trypanosomes contained a large number of inclusions. This

accumulation is well shown by the figures which demonstrate increase both in the numbers of inclusions and of affected trypanosomes, but the observer gains an even more impressive sense of this accumulation from the striking increase in size and refractility of the individual inclusions. From one to three inclusions occurred in the posterior end of the trypanosome but they were more numerous in the anterior end, often arranged in one or two rows like strings of beads. They were particularly numerous in posterior nuclear forms giving the appearance by their volume (in this strain) of having forced the nucleus over to the posterior end.

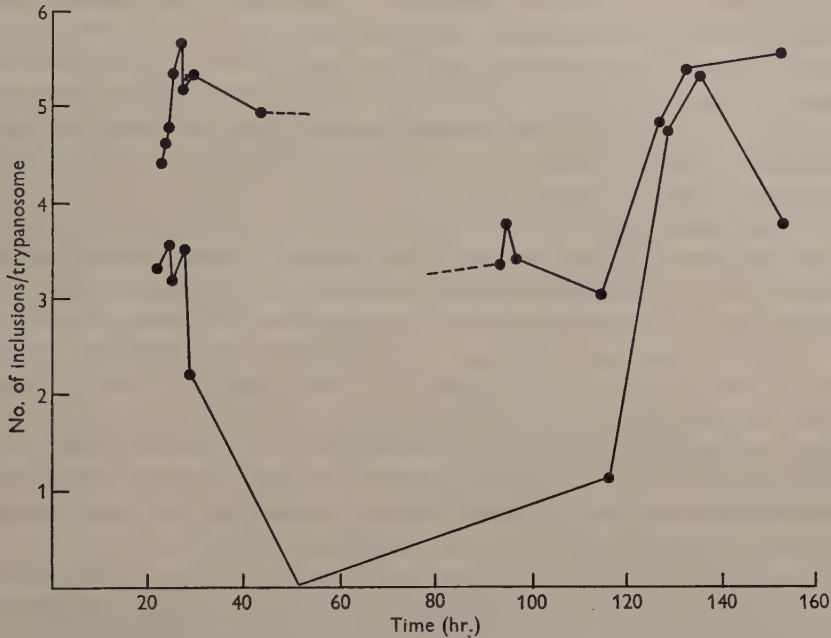


Fig. 3. *Trypanosoma rhodesiense*. 'Maun' strain. Graph showing the mean number of inclusions/trypanosome based on the infection in six rats. The upper curve was calculated by excluding the trypanosomes which contained no inclusions and shows that in the first 40 hr. of infection the individual trypanosomes (presumably derived from the original inoculation) did not lose their inclusions but were themselves eliminated from the circulation. The lower curve, based on the total number of trypanosomes, shows the abrupt fall in inclusion content at the beginning of the infection, rising to a maximum before the crisis. In counting the inclusions an arbitrary maximum of ten inclusions was set for each trypanosome since this seemed to be the greatest number which could be counted with accuracy in the confined space of one organism: the peak figures for inclusion content are lower, therefore, than they should in fact be.

Posterior nucleus forms were very numerous during the few hours preceding the crisis. During the crisis the blood contained numerous refractile globules of the size and appearance of the cytoplasmic inclusions of the stumpy forms.

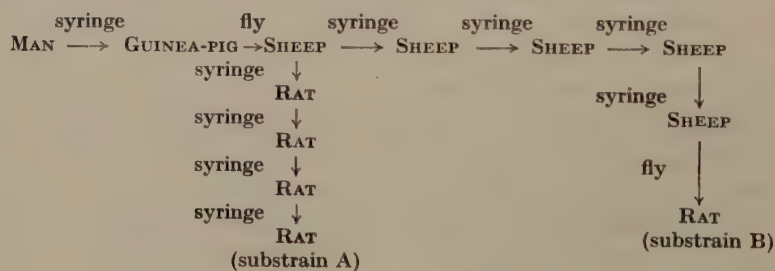
After the crisis, which never completely cleared the blood, the infection began to increase within 2-3 days. The numbers of long thin and short stumpy forms remained approximately equal and no further crisis was observed. The observations of the infection following the crisis are, however, tentative since

no exhaustive study was made of this phase of the infection. The rats survived 29 ± 8.7 days and death was associated with, and may have been caused by, allergic phenomena, i.e. swelling at the joints and nasal passages. During twelve passages in rats there was no noticeable change in the appearance of the organism or in the survival time of the rats, but the rapid initial development of the infection did not show itself until after three passages in rats.

Strain 2. 'SS 57' strain received from Dr J. R. Baker of E.A.T.R.O., Uganda, had been isolated from man. The infection was contracted on the shore of Lake Victoria between Majanji and Mfangano Island, and the fly was presumed to be *Glossina pallidipes*. I examined the infection in rats infected from a guinea-pig that had been infected from the patient. The appearance of the trypanosomes (see Pl. 1, fig. 6) was in contrast with those shown by strain 1. The infection began in the same way with long thin forms quickly changing over to short stumpy ones with gradual rise in the number of inclusions, but inclusions were more numerous, smaller and less refractile than those shown in strain 1.

It was seldom possible to count the inclusions in any one trypanosome or to declare in some instances with certainty whether a trypanosome contained inclusions or not. It was impossible therefore to make counts such as those shown in Figs. 1-3. Generally long thin forms were free from inclusions and short stumpy forms contained numerous inclusions, but exceptions from this rule occurred more frequently in strain 2 than in strain 1: posterior nuclear forms were present late in the infection but were less frequent in this strain, inclusions were equally small in these forms and there was no appearance of the nucleus having been pushed posteriorly. There were no signs of any immune reaction to the very acute infection produced by this strain, no crisis, and no allergic phenomena. The rats survived 9 ± 2.8 days.

Strain 3. 'Vom strain'. This strain was isolated in Bechuanaland 17 months before my observations were made. The history since isolation was as follows:



The observations which were made on 2 and 3 February 1955 were as follows:

Substrain A showed a very heavy infection (c. 100 trypanosomes/ $\times 600$ field). The trypanosomes were monomorphic and without inclusions except that c. 1% of the trypanosomes were of the broad stumpy form with heavy inclusions.

Substrain B showed a heavy infection (c. 20 trypanosomes/ $\times 600$); rats infected at the same time showed different rates of infection but identical

appearances. Long thin forms and short stumpy forms were present in approximately equal numbers and the appearance of granules and their distribution between the types of trypanosomes were identical with those shown in strain 1 as shown in Pl. 1, fig. 5.

Strain 4. Origin unknown. When first observed it had been maintained by syringe passage in laboratory rats for about 1 month; it was still polymorphic, the limits of which are shown in Pl. 2, fig. 7. One year later the strain was fully monomorphic and without inclusions.

Numerous other strains of *Trypanosoma rhodesiense* have been examined in various laboratories; all caused acute disease, the rats or mice surviving up to 5 days. The trypanosomes were monomorphic and were without inclusions: the same or similar strains in guinea pigs were more chronic and usually contained inclusions: polymorphism was variable or absent.

(14) *Trypanosoma gambiense*. I have seen a single trypanosome in the cerebrospinal fluid of a patient with sleeping sickness in the Gambia and three trypanosomes in a mouse infected from a patient from Sierra Leone. Apart from the fact that these four trypanosomes contained multiple inclusions I cannot report on the morphology of inclusions in this species. Strains that I examined adequately had previously undergone multiple syringe passage and had acquired characteristics similar to those acquired by other species of polymorphic trypanosomes treated in this way.

Group V (trypanosomes of cold-blooded vertebrates)

(15) *Trypanosoma rotatorium* Meyer, 1843. The leaf-like phase of this trypanosome was observed in two frogs (*Rana esculenta*) which had been imported from Europe. Only three specimens were seen, one of which is shown in Pl. 3, fig. 8. In each trypanosome rows of inclusions were arranged between the striae; no stained preparations were obtained.

(16) *Trypanosoma danilewskyi* Laveran & Mesnil, 1904. Four out of twelve common carp (*Cyprinus carpio*) from a pond in Essex were found to be infected; the infection was scanty and an adequate study was made only of four individual trypanosomes. Inclusions were present in these large, active, refractile trypanosomes but were often inconspicuous (see Pl. 3, fig. 9; the difference in overall density between the organisms shown in figs. 9 and 10 is not likely to be significant but probably reflects differences in the setting of the phase contrast microscope). A constant feature was a single inclusion or group of inclusions in the region of the kinetoplast.

DISCUSSION

These inclusions in trypanosomes are usually referred to as 'volutin granules', but it is probably better to call them simply 'inclusions' as this cannot lead one to suppose that their chemical nature is defined. Cytochemical evidence (Van den Berghe, 1942) suggests that they contain ribonucleic acid, but as methods available at present are clearly inadequate for this type of work (see section on Methods), this comparative study has been made solely on the refractility of the inclusions and their superficial appearance.

In spite of deviations, the overall consistent appearance of these inclusions leads me to suppose that I have been observing a single phenomenon in the different species of trypanosomes in the circulation of warm-blooded animals; the scantiness of evidence in connexion with trypanosomes of cold-blooded animals must exclude them for the present from the discussion.

The phenomenon studied consists of the following features. The appearance of spherical refractile bodies in the cytoplasm which vary in size from the limits of microscopic resolution to $4\ \mu$ across and the larger the inclusions, the less refractile appears the rest of the cytoplasm. These refractile bodies have no apparent connexion with the nucleus; in some species, especially of the *lewisi* group, they form most readily in the region of the kinetoplast, but in the *brucei* group they are most frequently anterior to the nucleus and are arranged like strings of beads. In some species, such as *Trypanosoma lewisi* and *T. cruzi* the refractile bodies do not stain with Romanowsky stains, in others (*T. nabiasi*, *T. grosi*) they stain readily; in *T. rhodesiense* they sometimes stain with ease taking on a deep violet colour, while at other times they show a pale eosinophilia, and at others they do not stain at all. The refractile bodies are not permanent cell structures and appear in the course of development of the trypanosome where they had not been visible before.

In the introduction to this paper I suggested (Ormerod, 1951) that the inclusions might be produced by a substance circulating in the blood of the host, absorbed by the trypanosome and able to disrupt its ribonucleoprotein into constituent protein and ribonucleic acid. No evidence either in proof or disproof of this hypothesis has emerged from the present work, but the results suggest that the phenomenon is in some way connected with an immune reaction on the part of the host and are therefore likely to be of more than theoretical importance in the study of trypanosomiasis. The evidence for this is as follows.

(1) The inclusions do not develop before the 5th day of the infection. (If, however, they are already present in the infecting organisms, they will appear on the first 2 days of the infection but will be absent on the second and third days.)

(2) In *Trypanosoma rhodesiense* strain 1, the maximum production of inclusions coincided with the crisis; the inclusion-filled trypanosomes were less motile, more fragile and in the last stages of the crisis were apparently moribund.

(3) *Trypanosoma rhodesiense* strain 1 produced heavy inclusions and was associated with immune phenomena in the host and a long survival time (29 days for rats). Strain 2 produced light inclusions, showed no immune phenomena and had a short survival time (9 days for rats).

(4) Chronic infections are usually associated with the formation of many inclusions; in acute infections the trypanosomes contain few inclusions or none at all. Exceptions to this rule are *Trypanosoma avium* and the *lewisi* group where a different mechanism appears to operate.

(5) In *Trypanosoma lewisi* the appearance of inclusions coincides with the initial check in growth of the organism ascribed by Taliaferro (1941) to a

growth-inhibiting factor ablastin. The adult forms which contain only one inclusion appear to be insensitive to this factor.

(6) In *Toxoplasma gondii* inclusions having similar appearance under phase contrast are undoubtedly produced by immune bodies. Analogy with this organism is sufficient to show that an immune reaction with this type of morphology is already known in the phylum Protozoa.

I do not propose to discuss further the possible relationship of inclusions to the immune reaction of the host, but to reserve this topic for a later publication.

A striking relationship, however, exists between polymorphism in trypanosomes and the appearance of inclusions. Inclusions without polymorphism occur in *Trypanosoma vivax* and *T. cruzi*, so that they do not necessarily go together, but they are closely associated in *T. rhodesiense* (presumably also in *T. gambiense* and *T. brucei*) and in *T. lewisi* and the other flea-transmitted trypanosomes of rodents which resemble it. In these two types of trypanosomes with widely differing life histories and behaviour, multiple inclusions occur only in the broad stumpy and intermediate forms. The long thin forms of *T. rhodesiense* are inclusion-free and those of *T. lewisi* have a single inclusion in the region of the kinetoplast. The broad inclusion-bearing forms in both species are associated in time (if not more directly) with an immune reaction in the host. The appearances suggest that in either case the long thin forms are resistant to this immune reaction; in *T. lewisi* the 'adult' forms are resistant to sera which are lethal to 'developmental' forms (Coventry, 1930), and in *T. rhodesiense* strain 1, the long thin forms divide readily and appear active while the short stumpy forms succumb at the crisis. The difference, however, lies in the reversal of the polymorphism, *T. lewisi* beginning the infection with a form that is susceptible to immune bodies and passing over to one that is resistant, whereas *T. rhodesiense* appears to begin its cycle in the blood with a resistant form passing over to a susceptible.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. *Trypanosoma lewisi*. 45th day of infection; freehand drawing of trypanosomes on agar as seen by phase-contrast. The adult forms show one (or less frequently two) inclusions posterior to the region of the kinetoplast.
- Fig. 2. *Trypanosoma lewisi*. 7th day of infection, broad forms showing multiple inclusions in the region of the kinetoplast.
- Fig. 3. *Trypanosoma grosi*. Inclusions appear either in the region of the kinetoplast or between the nucleus and the kinetoplast.
- Fig. 4. *Trypanosoma cruzi*. The body of the trypanosome is crammed with inclusions; the position of the nucleus is shown by the area without inclusions.

PLATE 2

- Fig. 5. *Trypanosoma rhodesiense*. 'Maun' strain. This strain, which produces a marked immune reaction in rats, shows large and very distinct inclusions, which are present only in broad and intermediate forms.
- Fig. 6. *Trypanosoma rhodesiense*. Strain SS57. Inclusions are smaller and less easily distinguished than in the Maun strain, usually confined to broad and intermediate forms. Little immune reaction was produced in rats.

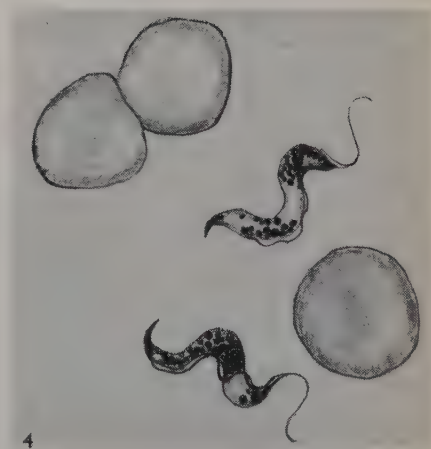
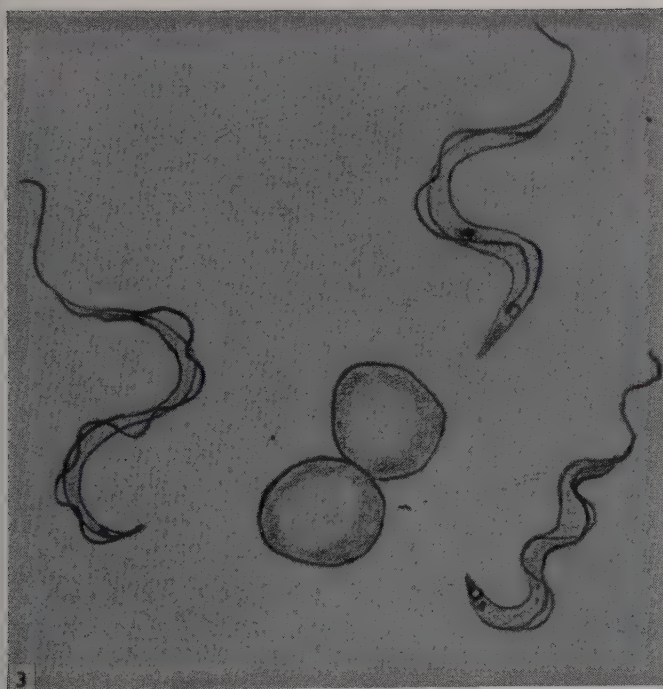
Fig. 7. *Trypanosoma rhodesiense*. Strain of unknown origin. Extreme forms: posterior nuclear form, very slow-moving and filled with inclusions. (The inclusions present in the undulating membrane are probably a unique feature of this individual organism): long thin form without inclusions. (The blunt posterior end was seen frequently in this strain.)

PLATE 3

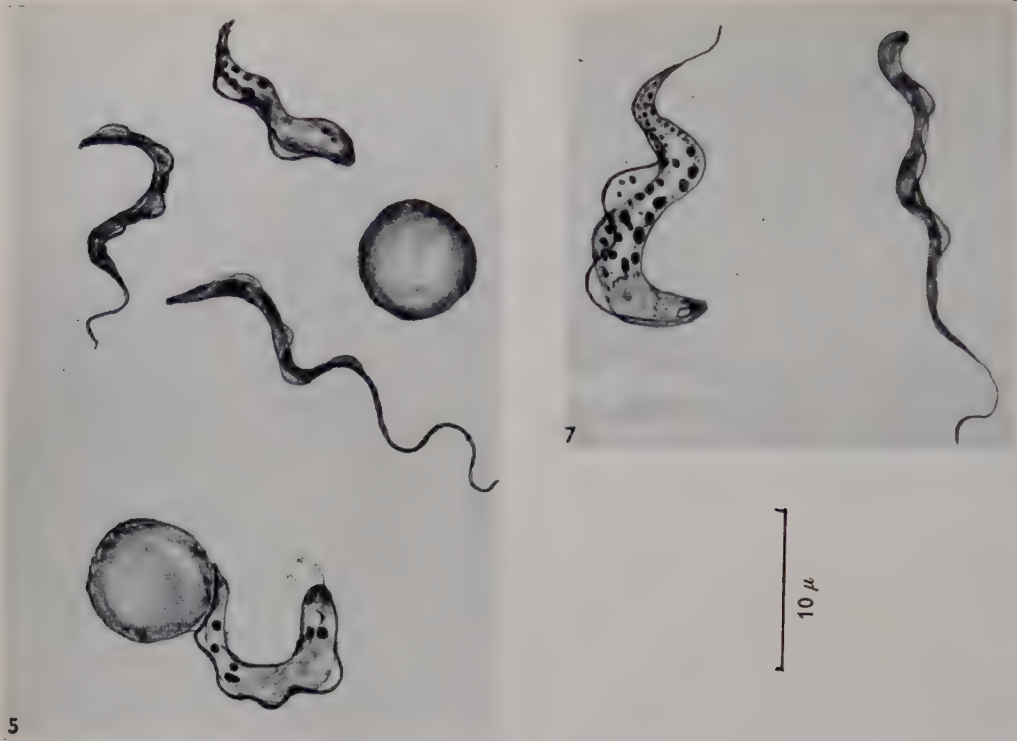
Fig. 8. *Trypanosoma rotatorium*. Inclusions are arranged in rows between what appear to be muscular striations.

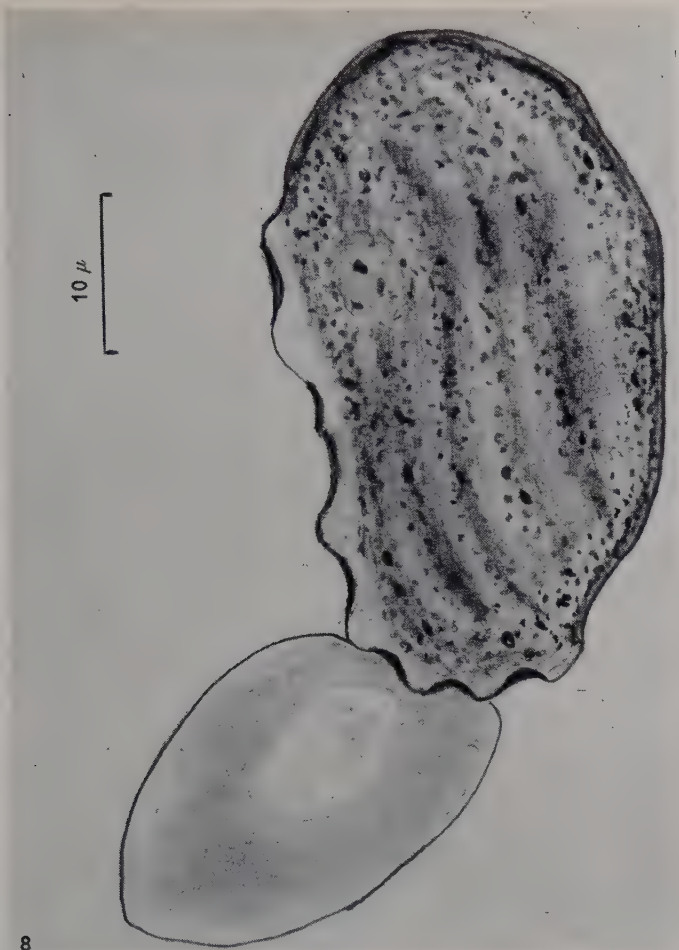
Figs. 9, 10. *Trypanosoma danilewskyi*.

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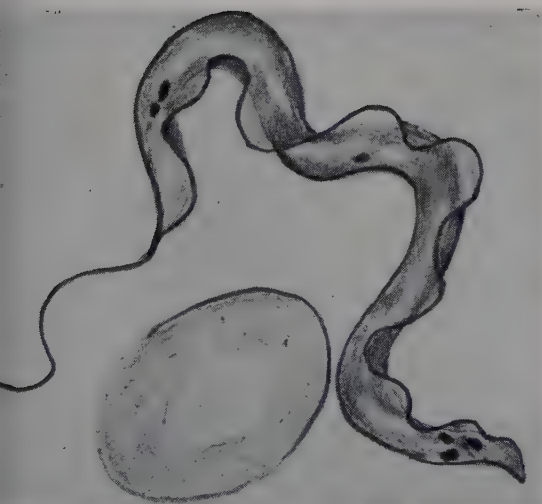


10 μ





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Bacteriocin-like Material Produced by *Pasteurella pestis*

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SUMMARY: Strains of *Pasteurella pestis* under certain conditions form bacteriocin-like material, for which the name 'pesticin' is proposed; it inhibits the growth of *P. pseudotuberculosis*. The conditions necessary for induction of pesticin formation are similar to those which lead to induction of formation of some known bacteriocins. Of 24 strains of *P. pestis* tested, all but one produced pesticin. A mutant of *P. pseudotuberculosis* which was resistant to pesticin produced by one strain of *P. pestis* was found to be resistant to pesticin produced by all the other strains.

Reciprocal antibiotic actions were first described by Gratia (1925) and Fredericq (1946), who found that certain strains of *Escherichia coli* produce antibiotic substances that act specifically on other strains of the same species. These substances were named colicins (Fredericq, 1946). Further work showed that similar substances were also produced by many strains of *Shigella* (Fredericq, 1948*a*; Fastier, 1949), some strains of *E. freundii* (Fredericq, 1947), and a few *Salmonella* strains (Fredericq, 1952; Hamon, 1955). It was shown in 1952 (Jacob, Siminovitch & Wollman, 1952), that it was possible to induce formation of colicin by irradiation of the bacteria under conditions similar to those which cause induction in lysogenic bacteria. The colicins differ in the extent and specificity of their activity and in their physical and chemical properties (Fredericq, 1948*b*; Goebel, Barry & Shedlovsky, 1956; Hamon, 1956; Ludford & Lederer, 1953). Analogous substances were found in two other families of bacteria: pyocin, produced by *Pseudomonas aeruginosa* (Jacob, 1954), and megacin, produced by *Bacillus megaterium* (Ivanovics & Alföldi, 1955, 1957). The present paper describes the formation of bacteriocin-like material by strains of *Pasteurella pestis*; the substance will be referred to as 'pesticin'.

METHODS

Organisms. The following strains were used: A strain of *Pasteurella pseudotuberculosis* No. 134 C (kindly supplied by Professor H. B. Maitland, Department of Bacteriology, Manchester) and a streptomycin-resistant mutant of this strain. Strains of *P. pestis*: 8775, 8776, 8777, 8779, Bombay, TJW (NCTC), TRU Schuetz, Ts otten, P4, Pf2, TSR, P5, P3, P2, P12, L37 (received from the Department of Bacteriology, Hebrew University, Jerusalem), EV76, 243, H2, Elis, 195, 1122 (kindly supplied by Professor G. Girard, Institut Pasteur, Paris). Kimberley strain P (received from Institute Oswaldo Cruz, Rio de Janeiro), strain P in a rough stage 'PR', a streptomycin-resistant mutant of

P. pestis strain TRU. A strain of *Shigella dysenteriae* (Shiga). A strain of *Escherichia coli* K12 (λ). A strain of *Pasteurella tularensis* strain Schu. Strains of *Salmonella typhimurium*, 156, 238, 243, 154, 246, 244.

Media. Tryptose broth (TS) consisted of Bacto Tryptose (Difco) 2% (w/v), 0.5% NaCl (w/v). Proteose peptone broth (Pr₃) consisted of Proteose peptone 3 (Difco) 2% (w/v), NaCl 0.5% (w/v), glucose 0.05% (w/v), Na₂HPO₄, 0.5% (w/v). Blood agar base (BAB) consisted of: blood agar base (Difco) 4% (w/v), defibrinated rabbit blood 3% (w/v). Tryptose agar (TSA) and proteose peptone agar (Pr₃A) were prepared as described for liquid media with the addition of agar (Difco) 2% (w/v). Phosphate buffer contained NaH₂PO₄ 5% (w/v), Na₂HPO₄ 5% (w/v) (pH=7). Trypsin solution: trypsin 1/300 (Nutritional Biochemical Corporation, Cleveland, Ohio) was dissolved in a buffer salt solution of the following composition (% w/v): NaCl, 0.8; KCl, 0.02; Na₂HPO₄, 0.115; KH₂PO₄, 0.02; MgCl₂·6H₂O, 0.01; CaCl₂, 0.01.

Conditions of cultivation. Stock cultures were maintained on BAB slopes. Broth cultures were started from 24 hr. blood agar slopes and incubated at 28°–30° for 20–24 hr. on a shaker.

Ultra-violet irradiation. The irradiation of 4 ml. samples in 9 cm. Petri dishes (less than 2 mm. layer of liquid) was carried out under constant agitation. A G15T8 hot Sterilamp (Westinghouse Electric Corporation, Bloomfield, N.J., U.S.A.) was used for irradiation. The intensity (measured with a SM-600 u.v. indicating meter for sterilamps) was 300 ergs sec.⁻¹ cm.⁻² at a distance of 87 cm. The same dose was used for irradiation of bacteria on solid media. After irradiation all further work was performed in dim light to avoid photo-reactivation.

Exposure to sonic vibration. Five to 10 ml. samples were exposed to sonic vibrations at 10 kc. in a 250 W. 'Raytheon' magnetostriction oscillator, Model DF 101 (Raytheon Manufacturing Co., Waltham, Mass., U.S.A.).

Optical density. The optical density of suspensions of organisms was measured with a Coleman Junior spectrophotometer at a wave length of 590 m μ .

Viable count. Samples (0.1 ml.) of an appropriate dilution of a culture were plated on BAB medium. The colonies were counted after incubation for 48 hr. at 30°.

Demonstration of pesticinogenic properties (two-layer method, Fredericq, 1954a). The bacteria tested for pesticin production were seeded in 1 ml. of Pr₃A on a basal medium (Pr₃A) layer. Immediately after the 1 ml. of agar solidified, a second layer of 10 ml. of Pr₃A was poured over the first and the plate incubated for 3 days at 30°. At the end of this period, when isolated colonies of the seeded culture appeared between the two layers of agar, the sterile surface of the upper layer was seeded evenly with the indicator strain, *Pasteurella pseudotuberculosis*. This was done by means of filter-paper which was soaked in a suspension of that strain. After 24 hr. further incubation at 37°, the indicator strain developed uniformly, except for some circular inhibition zones situated above the active colonies.

Pesticin titration. The pesticin was titrated by spotting drops (0.01 ml.) of a series of dilutions of the preparation to be titrated, on the surface of a plate

seeded with the indicator strain. The indicator plates were prepared some minutes before the titration by soaking a filter-paper in a suspension of the strain and leaving the paper on the plates for 5 min. The plates were then incubated at 37° for 24 hr. In this way a series of zones of decreasing inhibition was obtained ranging from complete inhibition through partial inhibition to normal growth. The highest dilution that gave a visible inhibition zone was defined as containing one arbitrary unit/ml.

RESULTS

Pesticin formation

The supernatant fluid from a well-aerated 48 hr. broth culture of *Pasteurella pestis*, grown at 30°, contained a substance (pesticin) which inhibited the growth of *P. pseudotuberculosis*.

After centrifugation of culture in the cold at 3000 rev./min. for 20 min. its supernatant fluid was treated as follows: (a) by addition of a few drops of chloroform to the supernatant, vigorous shaking for 10 sec., decantation followed by aeration of the liquid for 20 min. at 37° (to remove the residual chloroform); (b) by addition of 500 µg. dihydrostreptomycin (sulphate)/ml. and using as indicator a streptomycin-resistant mutant. Checks for sterility were performed after each treatment. These treatments did not destroy pesticin activity. Filtration of the supernatant fluid through ultra-fine sintered glass filters sometimes decreased the inhibitory effect.

When material from the agar surface of an inhibitory zone was transferred (bacteriological loop) to another plate with an indicator strain, no inhibition was seen. The inhibiting agent could not be transferred in series.

Transmission of pesticinogenic properties. By means of the two-layer method it was found that each bacterium in a population of a pesticin-producing strain was capable of transmitting this property to its descendants (Pl. 1).

Influence of media on activity. Experiments showed that the media on which pesticin was titrated considerably influenced the results. While one batch of TSA gave very good results, a different batch did not show any inhibition of *Pasteurella pseudotuberculosis* growth. No differences were found between different batches of Pr₃A. The media on which pesticin was titrated were always adjusted to pH 7.

In liquid media inhibition of *Pasteurella pseudotuberculosis* growth by pesticin was observed but no quantitative results could be obtained. All pesticin titrations described in this paper were performed on solid media (see 'Methods').

Temperature influence on pesticin activity. The activity of pesticin was 20 times higher when tested at 37° than at 30°. Table 1 shows the respective activities obtained in the titration of pesticin on plates incubated for 24 hr. at 37° and 30°.

It should be noted that *Pasteurella pseudotuberculosis* grows more abundantly at 37° than at 30° (unlike *P. pestis*).

As the pesticin was added directly on the indicator bacteria (*Pasteurella pseudotuberculosis*) and the presence and not the diameter of the inhibition zone was considered, no allowance was made for the variation in diffusion rate in agar resulting from differences in temperature.

Table 1. *Temperature influence on pesticin activity (activity expressed in arbitrary units)*

Samples of pesticin	Activity measured at 37°	Activity measured at 30°
1	400	20
2	1,000	50
3	2,000	75
4	10,000	200
5	8,000	300

Production of pesticin by different strains and spectrum of activity. Out of 24 strains of *Pasteurella pestis* tested, only strain TRU failed to produce pesticin. This strain was not sensitive to the pesticin produced by other strains of *P. pestis*. Attempts to transduce the pesticinogenic properties of other strains of *P. pestis* to strain TRU, by growing the two strains together, were not successful (cf. Fredericq, 1954b). *P. tularensis* strain Schu., *Escherichia coli* K12 (λ), *Shigella dysenteriae* (Shiga), and 6 strains of *Salmonella typhimurium*, were not sensitive to pesticin. A *P. pseudotuberculosis* mutant, resistant to the pesticin produced by one strain of *Pasteurella*, was resistant to the pesticin produced by all other strains.

Chemical and physical properties of pesticin

Nature of pesticin. Pesticin is sensitive to proteolytic enzymes. Its activity is completely destroyed by incubation with 0.025 % (w/v) trypsin for 20 min. (1/300 NBC) at 37° (pH 7). It has a relatively high molecular weight since it diffuses slowly in agar and does not pass through cellophan.

Table 2. *Effect of heat on pesticin activity (pesticin activity expressed in arbitrary units)*

Original value 2000		
Time of heating	In boiling water	At 60°
1 min.	100	—
3 min.	50	—
5 min.	50	—
15 min.	2	—
1 hr.	—	50
2 hr.	—	25
3 hr.	—	2

Heat. Pesticin is thermolabile, its activity is decreased by more than 95 % through heating for 5 min. in boiling water. A similar effect is obtained by heating to 60° for 3 hr. Table 2 gives an example of the effect of heat on pesticin. Most of its activity is lost after one day at room temperature (30°)

and it cannot be kept for more than a few days in an ordinary refrigerator. Storage for 3 months at -20° did not decrease its activity.

pH. Pesticin is stable between pH 6 and 8; outside this range its activity diminishes. Table 3 shows the effect of variation in the pH of the media (Pr_3), containing the pesticin, on its activity. The media containing pesticin were kept for 2 hr. at 37° at various pH values. Part of the medium was brought back to its original pH (7), while the remainder was kept at the altered pH value. Titration was then carried out in the usual manner on both parts. Where the titration of pesticin was carried out at a pH different from 7, the dilution solution (Pr_3) was adjusted to the identical pH value. Where the pH which was outside 6–8 range was brought back to 7, the activity did not return to its previous value.

Table 3. *Activities of pesticin at different pH values*
(activity measured in arbitrary units of pesticin)

pH	Titration without further alteration in pH	Titration after readjustment to pH 7
12	2	2
11	100	100
10	800	800
9	1000	1000
7	2000	2000
5	1000	1000
4	400	400
3	100	100
1.5	50	50

U.v. irradiation. Pesticin is very resistant to u.v. irradiation. No decrease in activity was detected after irradiation for 75 min. at $300 \text{ erg. sec.}^{-1} \text{ cm.}^{-2}$.

Adsorption. Pesticin is completely adsorbed by Seitz filters and partly by sintered glass filters.

Induction of pesticin biosynthesis

The formation of pesticin by strains of *Pasteurella pestis* could be induced by u.v. irradiation. The conditions of pesticin induction were found to be similar to the conditions for induction of other bacteriocins in bacteriocinogenic bacteria (Kellenberger & Kellenberger, 1956; Hamon & Lewé, 1955).

Liquid cultures (24 hr.) of *Pasteurella pestis* in media TS or Pr_3 , of optical density ≥ 90 , did not contain pesticin. When these cultures were exposed to sonic vibrations for 20 min., 88 % of the bacteria were disintegrated, but no pesticin was found in the culture fluid. A similar medium containing pesticin, subjected to the same degree of sonic vibration for 20 min., did not show any decrease in the activity of pesticin.

When 24 hr. cultures of *Pasteurella pestis* were irradiated with a suitable dose of u.v. radiation pesticin appeared in the medium. An example of pesticin formation after u.v. irradiation is shown in Fig. 1. In this and similar

experiments it was observed that after irradiation, pesticin appeared in the medium after 15 min. at 37°, and only after 30 min. at 30°. (The optimum temperature for growth of *P. pestis* is 30°.)

The amount of pesticin liberated into the medium increased with time; at 37° it reached the maximum 2 hr., and at 30° in 3 hr. after irradiation. The irradiation itself caused a decrease in viable bacterial count of 50 % (irradiation for 90 sec.). No decline in bacterial count during incubation of the irradiated culture was detected.

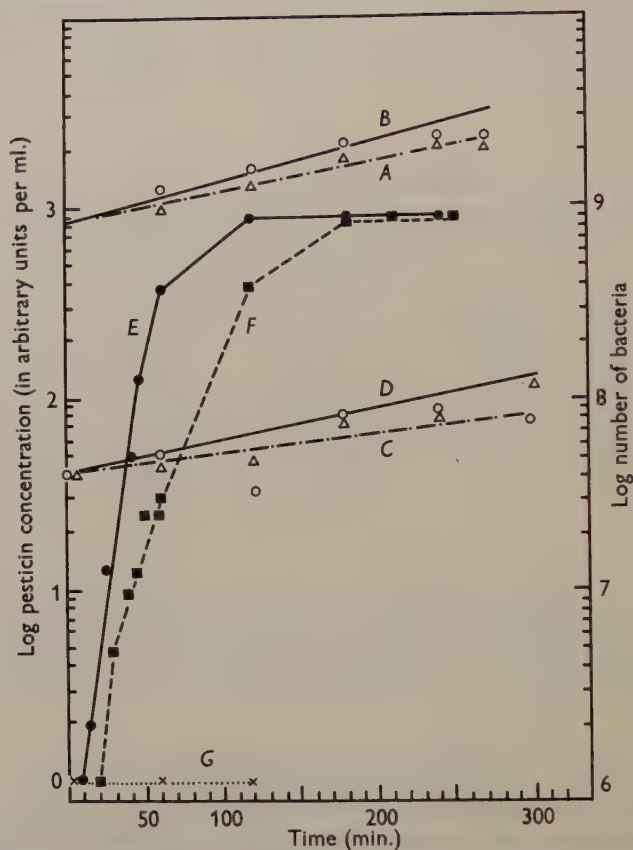


Fig. 1. Pesticin formation after u.v. irradiation of a culture of *Pasteurella pestis*, strain Kimberley (P), grown and irradiated in Pr₃ medium. The times of irradiation were zero and 90 sec. optical density 98. Pesticin concentration expressed in arbitrary units per ml. A, Count of non-irradiated bacteria at 37°; B, count of non-irradiated bacteria at 30°; C, count of irradiated bacteria at 37°; D, count of irradiated bacteria at 30°; E, pesticin formation at 37°; F, pesticin formation at 30°; G, pesticin formation at 40°.

Irradiation of organisms on solid media

Microcolonies of *Pasteurella pestis* on medium Pr₃A formed after incubation for 20 hr. were irradiated for 90 sec. and then tested for pesticin formation by the double-layer method. Inhibition zones appeared without visible bacterial growth of *P. pestis* (Pl. 1, fig. 2). No inhibition zones were formed when the

bacteria on agar were killed by exposure to chloroform vapour immediately after irradiation. Exposure of the bacteria to chloroform vapour 2 hr. after irradiation did not affect the formation of the inhibition zones.

Pesticin formation in buffer

Pasteurella pestis, grown for 24 hr. in medium Pr₃, was washed twice with phosphate buffer and then irradiated (90 sec.). Immediately after irradiation one part of the bacterial suspension was diluted 1/4 in Pr₃ medium, and another part was similarly diluted in phosphate buffer and both suspensions were incubated at 37°. After 2 hr. of incubation, pesticin was formed only in the Pr₃ broth; there was no formation of pesticin in phosphate buffer.

Inducibility of different strains of Pasteurella pestis. U.v. irradiation induced the production of pesticin by all the strains which could produce it spontaneously. Strain TRU which did not form pesticin spontaneously, did not produce it after u.v. irradiation.

DISCUSSION

The production of antibiotic material which inhibits the growth of *Pasteurella pseudotuberculosis* seems to be a common phenomenon in the species *P. pestis*; 23 out of 24 strains tested (or 22 out of 23 strains, if strain EV and ELIS are identical; Girard, 1957) produced pesticin.

The properties of pesticin allow it to be distinguished from classical antibiotics and to be classified with other bacteriocins, i.e. protein-like substances produced by bacteria which inhibit specifically related strains. The formation of some bacteriocins can be induced by ultraviolet irradiation. While generally as a result of induced formation of other bacteriocins bacteria are lysed (Jacob *et al.* 1952; Ivanovics & Alföldi, 1957; Jacob, 1954), pesticin formation after u.v. irradiation is not accompanied by total lysis. It is possible that only a very small proportion of the population produces pesticin and undergoes lysis. The methods used here (viable count and spectrophotometry) are not sensitive enough to detect a change in less than 10% of the population. It is therefore possible that production of pesticin is accompanied by lysis of a small proportion of the population. According to some authors (Fredericq, 1954c; Hamon & Lewé, 1955; Kellenberger & Kellenberger, 1956), however, induction of colicin formation does not result in lysis, unless the strains are also lysogenic. Kellenberger & Kellenberger (1956) examined 63 colicinogenic strains of *Escherichia coli* and concluded that strains that were only colicinogenic did not undergo lysis after u.v. irradiation, while a complete or partial lysis was always due to the presence of developing bacteriophage in the bacteria. After irradiation of colicinogenic, but not lysogenic bacteria, a residual growth of the bacteria was observed, and this was followed by an apparently stationary phase in the growth, while the production of colicin continued (Hamon & Lewé, 1955). In the case of pesticin formation following irradiation, no stationary phase in the growth curve was observed. The bacterial count increased during pesticin formation. It is quite possible that the expected plateau was masked by the growth of non-induced bacteria.

When bacteria on agar plates are induced by u.v. irradiation to form pesticin, they do not form colonies. This may indicate that pesticin synthesis is a lethal biosynthesis, and that the growth of organisms in irradiated liquid medium might be due to the growth of organisms which were not affected by irradiation.

We are indebted to Miss K. Schajevitz for excellent technical assistance.

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Fig. 1

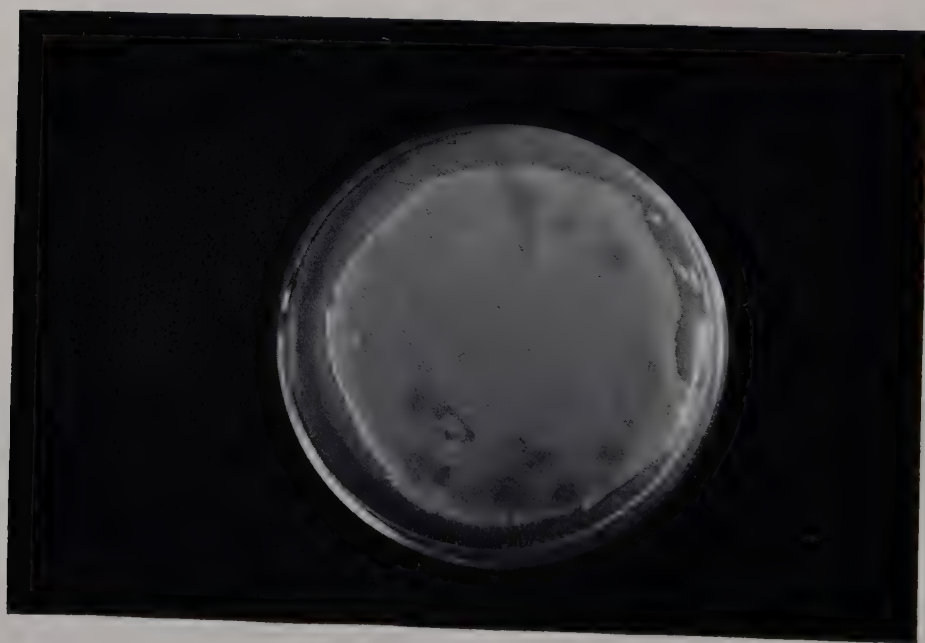


Fig. 2

R. BEN-GURION & I. HERTMAN—PESTICIN. PLATE 1

(Facing p. 297)

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EXPLANATION OF PLATE

- Fig. 1. Inhibition zones of *Pasteurella pseudotuberculosis* above colonies of *P. pestis*.
- Fig. 2. Inhibition zones of *Pasteurella pseudotuberculosis* produced by irradiation of micro-colonies of *P. pestis*.

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Nutritional Studies of *Zygorhynchus* Species

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SUMMARY: Nutritional experiments were carried out dealing with: (1) the utilization of carbohydrates; (2) the nitrogen source; (3) the action of different mineral salts and their interaction. The results showed that the strains of *Zygorhynchus* used grew well at 25° at pH 8.0 when buffered with nutritionally inert tris buffer and incubated for 12-14 days. Statistical analysis of the results showed that under the given conditions optimal growth measured as mg. dry wt. was obtained in liquid media containing 4% (w/v) glucose; 0.1 M tris buffer; 0.00625 M-(NH₄)₂SO₄; 2 p.p.m. Fe, Zn and Mn; with the following optimal conditions for the different strains specified: (i) for *Z. moelleri* CMI 0.002 M-KH₂PO₄; 0.012 M-MgSO₄.7H₂O; (ii) for *Z. moelleri* Baarn 0.002 M-KH₂PO₄; 0.006 M-MgSO₄.7H₂O; (iii) for *Z. vuillemini* CMI 0.002 M-KH₂PO₄; 0.003 M-MgSO₄.7H₂O; (iv) for *Z. vuillemini* Baarn 0.004 M-MgSO₄.7H₂O. There was a balance between the possible pairs of the salts from KH₂PO₄, MgSO₄.7H₂O and (NH₄)₂SO₄ (in one case only between KH₂PO₄ and MgSO₄.7H₂O). There was an optimal balance between all the salts for two strains. The best carbon sources were glucose, fructose, mannose, xylose and arabinose. The best sources of nitrogen were L-glutamine, L-asparagine, glycine, (NH₄)₂SO₄.

In 1953 Burges & Fenton observed that in the acid soils of East Anglia *Zygorhynchus vuillemini* was abundant below 10 cm., but relatively scarce in the surface regions; this distribution was correlated with a high tolerance to carbon dioxide. Several workers have studied the effect of different media on the reproductive processes of *Zygorhynchus* spp. but no detailed account of the general mineral nutrition of members of this genus has been given. The purpose of the present paper is to report on a nutritional study of some different *Zygorhynchus* spp.

METHODS

The following organisms were used. *Zygorhynchus moelleri* and *Z. vuillemini* from the Commonwealth Mycological Institute (CMI) which are zygospore-producing strains; *Z. moelleri* and *Z. vuillemini* from the Central Bureau voor Schimmelcultuur, Baarn (Baarn), which were non-zygospore-producing strains. Throughout this paper the following abbreviations are used: CMI strains are designated MC and VC, respectively, while the Baarn strains are designated MB and VB, respectively.

Single spore cultures of these fungi were kept as stocks in 6 in. × $\frac{3}{4}$ in. test tubes on potato glucose agar at 0° and subcultured as required. No alteration of morphological and cultural characteristics of these fungi occurred during the

period of the experiments. Exploratory experiments with VC showed that in phosphate-containing media a good source of inorganic nitrogen was $(\text{NH}_4)_2\text{SO}_4$, but the use of this substance led to an accumulation of H_2SO_4 in the medium (see also Machlis, 1953; Fothergill & Yeoman, 1956), to a consequent decline in pH value, and to the phosphate acting as a buffer as well as a nutrient. The use of an inert buffering agent was thus desirable. Tris buffer (2-amino-2-hydroxymethylpropane-1:3-diol) was found to be suitable.

In the nutritional experiments inoculations were made into a final volume of 25 ml. sterile culture fluid in Pyrex Erlenmeyer flasks (150 ml.) and incubated at 25°. Sterile phosphate solution was added aseptically from a glass syringe after autoclaving in order to avoid precipitation. The inoculum was a suspension of spores and mycelial fragments made by adding 10 ml. sterile distilled water to a suitable agar slope, shaking and decanting the liquid; 1 ml. of which was then added to each culture flask of liquid medium. It was found that the exact amount of the inoculum did not appreciably affect the amount of growth within the limits of the experiments.

All experiments were carried out on five replicates unless otherwise stated. After the growth period the mycelia were filtered on to tared Whatman No. 5 filter-papers and washed with distilled water. The mycelium with the filter-papers was then dried overnight at 85°, cooled in a desiccator and weighed; results are expressed as mg. dry wt. mycelium/flask. It was not found practical to separate mycelium from filter-paper after filtration, and as these filter-papers lose 7% of their original weight on drying under the above conditions, appropriate allowance was made for this. For preliminary experiments the following basal defined liquid medium A was used: 4% (w/v) glucose; 0.004 M- KH_2PO_4 ; 0.002 M- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.0125 M- $(\text{NH}_4)_2\text{SO}_4$; Zn, Fe and Mn at 2 p.p.m.

When tris buffer to 0.1 M was used the mineral constituents had be modified for maximum growth in all cases to contain 0.002 M- KH_2PO_4 ; 0.006 M- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; and 0.00625 M- $(\text{NH}_4)_2\text{SO}_4$; the complete modified medium containing tris buffer will be referred to as medium B. Preliminary experiments indicated that the temperature for maximum mycelial yield was 25°; a decrease in dry weight began on the 12th day of incubation with organisms MC and VC, and on the 14th day for organisms MB and VB.

RESULTS

Carbon requirements

The utilization of different carbon compounds by the strains of *Zygorhynchus* used was determined after 7-, 14- and 28-day growing periods. The basal medium B was used, but glucose was replaced by other carbon compounds at 15 g./l. Caramelization of the sugars was largely avoided by careful autoclaving, although this usually occurred to a small degree. The pH value of the resultant solutions after the addition of carbohydrates was adjusted to 8.0 which is a favourable value for these fungi. The following carbohydrates were used in this experiment, glucose, fructose, mannose, galactose, sorbose,

arabinose, xylose, maltose, lactose, sucrose and raffinose. The results showed that there was a large variation among the organisms tested in their ability to use, and their rate of utilization of, the different carbohydrates. After 7 days of incubation of organism MC only glucose, fructose and mannose were utilized to any large extent, but after 28 days yields of about 170 mg. dry wt. mycelium were obtained, and also with galactose or mannose; yields of mycelium with xylose and arabinose were also high. A similar pattern of utilization obtained also for organism MB but with a smaller yield of mycelium. With sucrose organism MB produced 141 mg. dry wt. after 28 days as against 24 mg. for organism MC. Incubation of organisms MC and MB for 7 days gave relatively small yields with all carbohydrates, but after 14 and 28 days all except sorbose, lactose and raffinose were utilized. The Baarn strains, unlike the CMI strains, utilized sucrose (125 mg. dry wt. mycelium as against 36 mg.). Maltose gave only 41 mg. dry wt. of mycelium with organism MB after 14 days but 132 mg. after 28 days incubation; organism MC gave 161 mg. dry wt. mycelium with arabinose, while organism MB gave only 35 mg. In general, both of the CMI strains gave consistently higher weights of mycelium than the Baarn strains.

Lilly & Barnett (1953) made a comprehensive study of the effect of sugars on the growth of a large number of fungi which included *Zygorhynchus vuillemini*. Their basal medium, however, differed from that used here and the pH value was considerably lower. A comparison is therefore not strictly accurate, but nevertheless may be made. In both sets of experiments glucose, fructose, mannose and galactose were good sources of carbon, while sorbose and lactose were the poorest sources. Organism VC was again exceptional as giving a high yield with arabinose.

Nitrogen requirements

In this experiment the effect of different sources of nitrogen was determined. The basal medium B was used with glucose and the nitrogen salt was varied but supplied at a nitrogen concentration equivalent to 0.00625M- $(\text{NH}_4)_2\text{SO}_4$. The pH value was adjusted to 8.0 in all cases. With all strains used there was a steady increase in nitrogen utilization in all its forms tested during 7-14 days of incubation; at 28 days there was less dry wt. of mycelium than at 14 days. In all strains the highest yields of mycelium were obtained with L-glutamic acid, L-asparagine or glycine (from 182 to 236 mg. dry wt. mycelium); $(\text{NH}_4)_2\text{SO}_4$, and NH_4NO_3 gave the next highest average yields (156-163 mg. dry wt. mycelium); while aspartic acid gave 121 mg. and KNO_3 only 76 mg. In general, as with the carbon requirement, the Baarn strains gave consistently lower yields of mycelium than those from the CMI. Thus these fungi fall into Robbins's (1937) second class according to their ability to utilize mineral nitrates, ammonium salts and organic forms of nitrogen.

Factorial experiments

The object of these experiments was to determine the effect of varying the concentration of the mineral salts and ammonium sulphate in the basal

medium. The factorial design and statistical analysis of the results should indicate whether or not a balance between the salts is necessary for high mycelial yields under the given conditions. The direct effect of the individual salts in the medium and the interaction between them is also determined. Medium B was used in these experiments; cultures were incubated at 25° for 12 days for CMI strains and for 14 days for Baarn strains. The results were expressed as mg. dry wt. mycelium average of four replicates in each case. The concentrations of the salts were fixed on the basis of halving and doubling them in the basal medium. Thus, KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $(\text{NH}_4)_2\text{SO}_4$ were each used at three concentrations and all possible combinations of them were set up. In any one experiment with any one strain 108 culture flasks were incubated. The initial pH value of all solutions was 7·8–8·0 and any tendency for a decrease in pH value was controlled by the tris buffer. The results are shown in Tables 1 and 2 and the analyses of variance in Table 3.

Table 1. *Growth responses of four strains of Zygorhynchus moelleri and Z. vuillemini to varying concentrations of KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $(\text{NH}_4)_2\text{SO}_4$*

Concentration of KH_2PO_4 : P_1 , P_2 , $\text{P}_3 = 0\cdot001\text{M}$, $0\cdot002\text{M}$, $0\cdot004\text{M}$; of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: Mg_1 , Mg_2 , $\text{Mg}_3 = 0\cdot003\text{M}$, $0\cdot006\text{M}$, $0\cdot012\text{M}$; of $(\text{NH}_4)_2\text{SO}_4$: N_1 , N_2 , $\text{N}_3 = 0\cdot003125\text{M}$, $0\cdot00625\text{M}$, $0\cdot0124\text{M}$.

A. *Z. moelleri* CMI (=MC) and *Z. moelleri* Baarn (=MB)

	P_1						P_2						P_3					
	Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3	
	MC	MB	MC	MB	MC	MB	MC	MB	MC	MB	MC	MB	MC	MB	MC	MB	MC	MB
	Mean dry wt. mycelium (mg./flask)																	
N_1	123	117	125	123	132	120	134	118	135	118	136	117	130	116	131	119	143	127
N_2	164	152	169	155	176	158	175	168	178	170	183	162	143	171	166	165	182	166
N_3	147	154	160	155	165	155	140	148	144	145	161	144	125	147	127	142	134	142

B. *Z. vuillemini* CMI (=VC) and *Z. vuillemini* Baarn (=VB)

	P_1						P_2						P_3					
	Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3	
	VC	VB	VC	VB	VC	VB	VC	VB	VC	VB	VC	VB	VC	VB	VC	VB	VC	VB
	Mean dry wt. mycelium (mg./flask)																	
N_1	142	128	134	122	128	121	150	135	145	122	143	122	144	136	141	124	140	119
N_2	144	136	141	137	141	136	151	132	151	137	151	130	147	132	144	139	145	130
N_3	139	125	139	122	139	119	145	125	149	123	142	119	141	137	142	123	142	120

With organism MC there were significant differences between the yields and all concentrations of salts used except between $0\cdot001\text{M}$ and $0\cdot002\text{M}$ - KH_2PO_4 . The highest yield was produced with P_2 , Mg_3 and N_2 , which gave 183 mg. dry wt. mycelium. With organism MB there was no significant difference in yields with any of the concentrations of either KH_2PO_4 or MgSO_4 . Ammonium sulphate at $0\cdot00625\text{M}$ gave 163 mg. dry wt. mycelium. The best growth was obtained with combination P_2 , Mg_2 and N_2 . With organism VC, except for the $0\cdot002\text{M}$ concentration where the increase of dry wt. was 10 mg., any

increases in dry weights were small but significant. The highest weight of mycelium produced was with P_2 , Mg_1 and N_2 . With organism VB all concentrations of $MgSO_4$ and $(NH_4)_2SO_4$ produced significant differences of yield, but with KH_2PO_4 the variation in yield was not large. The highest yield of mycelium with organism VB was produced with P_3 , Mg_1 and N_2 . With all strains 0.00625M- $(NH_4)_2SO_4$ gave the highest yields. The analyses of variance show that all first-order interactions were significant except that between KH_2PO_4 and $MgSO_4$; with organism VC that between $(NH_4)_2SO_4$ and $MgSO_4$ was significant only at the lower 19:1 odds. It is evident that with all four strains of *Zygorhynchus* used the concentration of ammonium sulphate and the interaction of this with KH_2PO_4 were important in order to obtain high yields of mycelium. In the second-order interactions between the three salts taken together only organism MC showed a high interaction at the 99:1 level of significance and organism MB at the 19:1 level.

Table 2. *Mean dry weights and growth responses of Zygorhynchus spp. after 12 and 14 days incubation at 25° on media with different concentrations of certain components*

Concentrations of salts as in Table 1, $KH_2PO_4 = P_1, P_2, P_3$; $MgSO_4 \cdot 7H_2O = Mg_1, Mg_2, Mg_3$; $(NH_4)_2SO_4 = N_1, N_2, N_3$. Difference required between weights for odds of 99:1 for strain MC=2.78; for strain MB=2.34; for strain VC=2.03; for strain VB=2.04.

Concn.	Organisms			
	MC	MB	VC	VB
	Mean dry weight mycelium (mg./flask)			
P_1	152	143	137	127
P_2	154	143	147	127
P_3	143	144	143	129
Mg_1	142	143	145	132
Mg_2	148	143	144	128
Mg_3	158	143	141	124
N_1	132	119	141	126
N_2	171	163	144	134
N_3	145	148	142	124
Mean	149.4	143.2	142.5	127.8

General mean=140.7 mg. dry wt. mycelium/flask.

In these factorial experiments KH_2PO_4 acted as a nutrient because its buffering effect was counteracted by the tris buffer which maintains the acidity at a constant value (Fothergill & Yeoman, 1956). The experiments indicated that a high degree of balance between the salts of the first order of interaction produced the highest yields of mycelium, but this balance was only absolute between all the salts in the second order of interaction in the case of organism MC and to a less extent of organism MB. Variation of balance in salt concentrations was thus shown between two different species of the same genus, i.e. between *Z. moelleri* and *Z. vuillemini*. In previous experiments of similar factorial design Talley & Blank (1941) and Fothergill & Asheroft (1955) showed that a correct balance between the mineral constituents of the

medium was essential in order to obtain a high yield of mycelium with two parasitic fungi, *Phymatotrichum omnivorum* and *Venturia inaequalis*, respectively. Fothergill & Raine (1954) showed that while a correct balance between salts was conducive to good growth, it was not of overriding importance with complementary strains of the saprophytic fungus *Mucor hiemalis*. Fothergill & Yeoman (1957) found with the saprophyte *Rhizopus stolonifer* that a balance existed between K_2HPO_4 and $(NH_4)_2SO_4$, but not between these and the other salts in the medium.

Table 3. *Analysis of variance (grouped) for Zygorhynchus strains MC, MB, VC and VB*

Required 'F' and 't' values taken from Snedecor's tables (1934). Sums of squares and mean squares are omitted from table.

Variance	D.F.	F required odds					
		Found				99:1	19:1
		Organism					
		MC	MB	VC	VB		
Total	107	—	—	—	—	—	—
(NH ₄) ₂ SO ₄	2	757.15	1317	28.3	108.5	4.88	3.11
KH ₂ PO ₄	2	65.157	0.41	11.6	4.7	4.88	3.11
MgSO ₄ ·7H ₂ O	2	111.667	—	7.07	679.5	4.88	3.11
(NH ₄) ₂ SO ₄ × KH ₂ PO ₄	4	58.071	39.4	7.1	5.1	3.56	2.49
(NH ₄) ₂ SO ₄ × MgSO ₄	4	6.847	3.749	2.62	23.5	3.56	2.49
KH ₂ PO ₄ × MgSO ₄	4	7.803	4.497	1.68	3.6	3.56	2.49
KH ₂ PO ₄ × MgSO ₄ × (NH ₄) ₂ SO ₄	8	10.595	2.612	1.1	1.3	2.74	2.06
Residual error	81	—	—	—	—	—	—

The strains of *Zygorhynchus* used in the present experiments show several major differences in their nutritional requirements and growth rates. The onset of decrease of dry wt. of mycelium varies with strain from 12 days with the non-sporing forms to 14 days in the sporing forms. *Zygorhynchus moelleri* and *Z. vuillemini* from the CMI were unable to utilize sucrose as source of carbon, while the strains from Baarn were able to use it to a marked extent. This may indicate the lack of invertase in the British strains. The Baarn strain of *Z. vuillemini* utilized arabinose, but all the strains used sorbose, lactose or raffinose to a small extent, or not at all. Lilly & Barnett (1953) found that sorbose had an inhibitory effect on the growth of several fungi. The hydrolytic products of lactose and raffinose were utilized by all the strains of *Zygorhynchus* tested. Thus it seems that these fungi lack the enzymes necessary for the hydrolysis of the higher sugars.

In the experiment on the nitrogen requirement only *Zygorhynchus moelleri* CMI used KNO_3 to any marked extent. The sporing strains of *Zygorhynchus* gave low mycelial yields with L-glutamine while the non-sporing strains gave high yields. The sporing strains also required higher Mg concentrations than the non-sporing strains; this may be correlated with Hawker's observation (1950) that magnesium concentration influences sporulation. Hence it may be

concluded that whilst these four strains of *Zygorhynchus* have similar basal nutritional requirements there are important differences between them.

The writers thank their colleagues Dr D. A. Evans, and Mr H. Campbell for their assistance with the statistical part of the paper.

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Selective Media for some *Pasteurella* Species

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SUMMARY: Three selective media are described for the isolation of *Pasteurella pestis*, *P. septica*, *P. pseudotuberculosis* and *P. haemolytica* from material heavily contaminated with other bacteria.

During studies carried out in this establishment on organisms of the genus *Pasteurella*, media were often required that would assist in the isolation of these organisms from material heavily contaminated with other bacteria. Selective media for this purpose previously reported by Drennan & Teague (1917), Meyer & Batchelder (1926) and Thal & Chen (1955) were found to allow the growth of many Gram-negative bacteria and were only of limited use. Recent screening tests on a large number of substances revealed the potential value of Novobiocin and Neomycin for the selective growth of certain *Pasteurella* spp. This report is concerned with the formulation of media using these substances for the detection of *P. pestis*, *P. septica*, *P. pseudotuberculosis* and *P. haemolytica*.

METHODS

Basal media. It was shown by Herbert (1949) that the aerobic growth of *Pasteurella pestis* on nutrient agar would occur quantitatively only when haemin, blood, or a reducing substance such as sodium sulphite, was added to the agar medium. The growth of pasteurellas was therefore compared, in the presence of selective substances, on peptone agar, tryptic digest of beef agar (TMA) and Difco tryptose agar (TA) with the addition of Fildes's (1920) digest of sheep's blood (PSB), sodium sulphite, or haemin. PSB was not necessary for the growth of some species but allowed the quantitative growth of all pasteurellas in higher concentrations of the selective agents than did haemin. Sodium sulphite in some instances diminished the selectivity of the media.

The growth of pasteurellas was better on TMA and TA than on peptone agar. Colonies of *Pasteurella pestis* and *P. haemolytica* were larger on TA but TMA produced the largest colonies of *P. septica*. *P. pseudotuberculosis* grew equally as well on TMA or TA. Within the range pH 6.8-7.8 the pH value of the medium had little effect on growth.

The basal media finally adopted were TMA for *Pasteurella septica* and *P. pseudotuberculosis*, and TA for *P. pestis* and *P. haemolytica* both at pH 7.6 sterilized in 8 oz. bottles at 120° for 20 min. The addition of 5% (v/v) peptic sheep's blood and of solutions of the selective agents in sterile distilled water, was made to the melted agar cooled to 50°. Petri dishes filled with 20 ml. amounts of the media were dried with the lids removed for 2 hr. at 37° to

remove surface moisture; they could be stored at 4° for at least 7 days without loss in selective properties.

Growth tests. The Miles & Misra (1938) technique was used to inoculate the basal and test media with pure cultures of pasteurellas and common contaminant organisms. In one series of experiments (Table 2) media were inoculated by a less accurate method. One drop of each pasteurella suspension was streaked radially on the medium with a Pasteur pipette, 10 strains being inoculated on each agar plate. The suspensions were diluted to give *c.* 100 colonies/drop. The number of colonies subsequently grown on the test media was estimated and compared with that obtained on the basal nutrient agar. Samples of the *Pasteurella* spp. were also added to suspensions in water, of soil (1 g./20 ml.), faeces (1 g./100 ml.) and straw (5 g./30 ml.) so that 0.1 ml. when plated on the media would yield *c.* 200 pasteurella colonies. Incubation was at 37° for 48 hr. for all species; *P. pestis* was also grown at 30°.

RESULTS

Growth of pure cultures. Selective substances were added individually to both basal media and tested with pure cultures of pasteurellas and contaminant organisms. There were marked differences in sensitivity to the selective agents among the *Pasteurella* spp. At concentrations showing some selective action, Novobiocin inhibited the growth of *P. septica*, partially inhibited *P. haemolytica* but allowed quantitative growth of *P. pestis* and *P. pseudotuberculosis*. Neomycin inhibited *P. pseudotuberculosis*, partially inhibited *P. pestis* but allowed normal growth of *P. septica* and *P. haemolytica*. Differences in sensitivity to potassium tellurite, Erythrocin and Tyrothricin were also found. The colony counts from some strains of each species were lowered in the presence of selective agents, but it was possible to adjust final concentrations in the media to a value which allowed normal growth of the maximum number of strains. *P. pestis* required an incubation temperature of 30°, higher temperatures resulted in irregularly sized colonies and smaller counts; 37° was preferred for growth of the other species. Colonies of pasteurellas were slightly smaller on the test media than on basal agar but were otherwise typical in appearance.

The selectivity obtained from individual additions to the media was inadequate for detection of pasteurellas from heavily contaminated material. Novobiocin allowed the growth of some Gram-negative bacteria, although it partially inhibited *Proteus vulgaris* and controlled its swarming. Neomycin effectively inhibited growth from Gram-negative contaminant organisms, including *P. vulgaris*, but did not affect *Streptococcus faecalis* or *Micrococcus* spp. The results are summarized in Table 1.

Certain mixtures of the selective substances were added to TMA and TA basal nutrient agar containing 5% PSB, and tested with pure cultures of *Pasteurella* spp. The results from a typical experiment are shown in Table 2. Medium 1, containing Novobiocin 10 µg./ml. + potassium tellurite 5 µg./ml. + Erythrocin 5 µg./ml. gave quantitative growth of *P. pestis* and *P. pseudotuberculosis* while inhibiting *P. septica* and *P. haemolytica*. Colonies of each

Table 1. Growth of pasteurellas and contaminant organisms on basal nutrient agar containing 5% PSB with added selective agents

Species	Basal agar	Selective agent									
		NOVO		NEO		TELL		ERYTH		TYRO	
		Quantity of selective agent (μg./ml.)									
		10	3	3	1.5	5	2	5	2	20	10
		Percentage of control count from basal media									
<i>Pasteurella pestis</i> (13)	TA	100	100	0-10	50-100	100	100	100	100	50-80	100
<i>P. pseudotuberculosis</i> (18)	TMA	100	100	0	0-10	100	100	100	100	100	100
<i>P. septica</i> (17)	TMA	0	0	100	100	100	100	30-50	50-70	100	100
<i>P. haemolytica</i> (6)	TA	20-50	80-100	100	100	20-50	80-100	30-50	50-70	50-80	100
<i>Proteus vulgaris</i> (4)	TMA	30 NS	50 NS	0	10 NS	S	S	S	S	S	S
<i>Escherichia coli</i> (4)	TMA	20	50	0	0	0	0	100	100	100	100
<i>Aerobacter aerogenes</i> (2)	TMA	0	0	0	0	0	20	100	100	100	100
<i>Alcaligenes faecalis</i> (6)	TMA	50	80	10	50	50	80	100	100	100	100
<i>Achromobacter</i> spp. (6)	TMA	20	80	30	50	50	80	100	100	100	100
<i>Streptococcus faecalis</i> (3)	TMA	0	0	100	100	100	100	0	0	0	30
<i>Staphylococcus aureus</i> (3)	TMA	0	0	0	30	100	100	0	0	0	0
<i>Micrococcus</i> spp. (3)	TMA	0	0	20	70	100	100	0	0	0	10
<i>Bacillus</i> spp. (5)	TMA	0	0	0	0	0	50	0	0	0	0

Figures in parentheses refer to the number of strains tested. NS=not swarming; S=swarming; NOVO=Novobiocin; NEO=Neomycin; TELL=potassium tellurite; ERYTH=Erythrocine; TYRO=Tyrothricin.

species grown were *c.* 1.2 mm. in diameter after 48 hr. of incubation and were of typical appearance.

The majority of strains of *Pasteurella septica* and two strains of *P. haemolytica* grew normally on medium 3 which contained: Neomycin 2.5 μg./ml. + Tyrothricin 10 μg./ml. *P. pestis* was partially inhibited on this medium and *P. pseudotuberculosis* completely suppressed. With potassium tellurite added to medium 3 (Table 2) the growth of *P. septica* only was obtained; colony counts from 5 of 17 strains, however, were then decreased (medium 2; Table 2). *P. septica* colonies were *c.* 1.5 mm. in diameter and of normal appearance.

Neomycin 1.5 μg./ml. + Novobiocin 2 μg./ml. in medium 5 (Table 2), suppressed the growth of *Pasteurella septica* and *P. pseudotuberculosis*, but allowed normal growth of *P. haemolytica* (with the exception of one strain, C. 1675). Six of the 13 strains of *P. pestis* were partially inhibited. The addition of Erythrocine 5 μg./ml. to medium 5 (Table 2) improved the growth of *P. pseudotuberculosis*, but inhibited *P. haemolytica* (medium 6; Table 2).

Tests with faecal and other suspensions

Media 1 to 7 (Table 2) were tested with suspensions of faeces, soil and straw, artificially infected with *Pasteurella pestis* 37, *P. septica* 9592, *P. pseudotuberculosis* 8315 and *P. haemolytica* B 1696. Medium 1 produced growth of *c.* 100 colonies of contaminant organisms/plate from faecal suspensions which produced confluent growth on basal nutrient agar. The colonies encountered on this medium were mainly of *Alcaligenes* spp. and were readily differentiated from pasteurella colonies. *P. pestis* and *P. pseudotuberculosis* were often isolated on medium 1 in pure culture from the soil and straw suspensions.

Pasteurella septica was readily isolated on media 2 and 3. Contaminant organisms growing on these media were mainly *Streptococcus faecalis* and

Micrococcus spp. These organisms were further restricted by increasing the concentration of Tyrothricin in the media but the recoveries of *P. septica* were then low. The other pasteurellas tested on media 2 and 3 did not grow from the artificially infected suspensions.

Pasteurella haemolytica was detected only on medium 5. Contaminant organisms growing on this medium were mainly Gram-positive bacteria and were readily differentiated from the pasteurella. *P. pestis* was also detected on medium 5 but in smaller numbers than on medium 1.

The number of pasteurella colonies growing on all media from the faecal suspensions was usually about 50 % of that expected from the size of inoculum,

Table 2. *Growth of pasteurellas on basal nutrient agar containing 5 % PSB with mixtures of selective agents added*

Species	Basal agar	Medium no.						
		1	2	3	4	5	6	7
		Selective agents added (μ g./ml.)						
		NOVO 10 TELL 5 ERYTH 5	NEO 2.5 TELL 2.5 TYRO 10	NEO 2.5 TYRO 10	NEO 2.5 NOVO 5	NEO 1.5 NOVO 2	NEO 1.5 NOVO 2 ERYTH 5	NEO 1.5 NOVO 2 TYRO 10
		Growth*						
<i>Pasteurella pestis</i>								
37	TA	+	—	tr.	tr.	+	+	+
Tjiwedij		+	—	sl.	sl.	+	+	+
EV 76		+	—	—	sl.	sl.	sl.	sl.
27		+	—	sl.	tr.	tr.	tr.	tr.
Java		+	—	tr.	tr.	tr.	+	+
Yokohama		+	—	sl.	tr.	+	+	tr.
Shasta		+	—	—	tr.	+	+	+
36		+	—	sl.	sl.	tr.	+	+
139 L		+	—	—	—	tr.	tr.	sl.
499559		+	—	tr.	tr.	+	+	+
F 9581		+	—	sl.	sl.	+	+	+
Hurni		+	—	—	—	tr.	tr.	sl.
TRU		+	—	tr.	tr.	+	+	+
<i>P. pseudotuberculosis</i>								
8487	TMA	+	—	—	—	—	tr.	—
1552		+	—	—	—	sl.	tr.	—
941		+	—	—	—	—	sl.	—
3569		+	—	—	—	—	sl.	—
8315		+	—	—	—	tr.	+	sl.
824		+	—	—	—	—	sl.	—
8604		+	—	—	—	—	tr.	—
3571		+	—	—	—	—	tr.	sl.
2478		+	—	—	—	—	sl.	—
8579		+	—	—	—	—	sl.	—
1894		+	—	—	—	—	sl.	—
2477		+	—	—	—	—	sl.	—
1779		+	—	—	—	sl.	sl.	—
AF		+	—	—	—	—	sl.	—
14/I		+	—	—	—	sl.	tr.	sl.
16/II		+	—	—	—	sl.	tr.	—
27/III		+	—	—	—	—	sl.	—
32/IV		+	—	—	—	—	tr.	—
		+	—	—	—	sl.	tr.	—

Table 2. (cont.)

Species	Basal agar	Medium no.						
		1	2	3	4	5	6	7
		Selective agents added ($\mu\text{g./ml.}$)						
		NOVO 10 TELL 5 ERYTH 5	NEO 2.5 TELL 2.5 TYRO 10	NEO 2.5 TYRO 10	NEO 2.5 NOVO 5	NEO 1.5 NOVO 2	NEO 1.5 NOVO 2 ERYTH 5	NEO 1.5 NOVO 2 TYRO 10
		Growth*						
<i>P. septica</i>								
9584	TMA	—	sl.	+	—	—	—	—
9592		—	+	+	—	—	—	—
1876		—	tr.	tr.	—	—	—	—
9581		—	sl.	+	—	—	—	—
8141		—	+	+	—	—	—	—
9577		—	+	+	—	—	—	—
9586		—	tr.	tr.	—	—	—	—
5868/0		—	+	+	—	—	—	—
9674		—	+	+	—	—	—	—
1140/0		—	sl.	+	—	—	—	—
7460		—	+	+	—	—	—	—
P 8		—	+	+	—	—	—	—
3195		—	+	+	—	—	—	—
4881		—	+	+	—	—	—	—
8283		—	+	+	—	—	—	—
1281		—	+	+	—	—	—	—
P 7		—	+	+	—	—	—	—
<i>P. haemolytica</i>								
A 1146	TA	—	—	sl.	tr.	+	—	tr.
B 1696		—	—	+	+	+	—	+
C 1576		—	—	sl.	sl.	sl.	—	sl.
C 1576/r		—	—	+	+	+	—	+
P 109		—	—	tr.	tr.	+	sl.	+
P 110		—	—	sl.	+	+	—	tr.

* + = 100 % of control count; tr. = 50 %–80 %; sl. = 10 %–50 %; — = no growth; NOVO = Novobiocin; TELL = potassium tellurite; ERYTH = Erythrocin; NEO = Neomycin; TYRO = Tyrothricin.

but on some occasions was only 5 % of the expected count. High recoveries, of the order of 80 %, were constantly found from the soil and straw suspensions. It was necessary to include Actidione 100 $\mu\text{g./ml.}$ or Mycostatin 200 units/ml. in the selective media for inhibition of the majority of moulds encountered from the suspensions. The growth of pasteurellas was not affected by either addition.

CONCLUSIONS

The selective substances used in the formulation of these media were the most valuable found by screening tests on more than 100 substances. It was not possible to devise one medium that would grow all *Pasteurella* spp. and maintain simultaneously a high degree of selectivity since the pasteurellas differed in sensitivity to most of the substances tested. The media finally adopted

represent a compromise between inhibition of the maximum number of contaminant organisms and the quantitative growth of the maximum number of pasteurella strains. This balance could be adapted to suit particular circumstances and for the detection of certain strains.

It should be noted that the results described are from examination of suspensions artificially infected with pasteurellas. Medium 1 has been used successfully, however, for the detection of *Pasteurella pseudotuberculosis* from faeces during a survey of over 400 'normal' guinea-pigs (Mr R. Cook, private communication). Each medium described has been used by the author for the separation of pasteurellas from contaminated cultures and for the isolation of these organisms from infected animals.

The three media which have been of the greatest use consist of TMA (for growth of *Pasteurellas septica* and *P. pseudotuberculosis*) or TA (for *P. pestis* and *P. haemolytica*) containing 5 % (v/v) peptic sheep's blood (PSB) with the following additions:

Medium 1. For *Pasteurella pestis* and *P. pseudotuberculosis*. Novobiocin 10 µg./ml. + potassium tellurite 5 µg./ml. + Erythrocine 5 µg./ml. + Actidione 100 µg./ml. *P. pseudotuberculosis* also grew on this medium when PSB was omitted.

Medium 2. For *Pasteurella septica*. Neomycin 2.5 µg./ml. + potassium tellurite 2.5 µg./ml. + Tyrothricin 10 µg./ml. + Actidione 100 µg./ml.

Medium 5. For *Pasteurella haemolytica*. Neomycin 1.5 µg./ml. + Novobiocin 2 µg./ml. + Actidione 100 µg./ml.

TMA was prepared by the method of Douglas, described by Fildes & McIntosh (1931), using Kobé no. 1 agar powder (Stafford Allen and Sons Ltd.). TA was prepared from Difco Bacto tryptose solidified with the agar powder noted above. The sources of the antibiotics used were as follows: Novobiocin from Glaxo Laboratories Ltd., England ('Biotexin' brand of Novobiocin sodium); Neomycin from Upjohn of England Ltd. ('Mycifradin' Neomycin sulphate); Erythrocine from Abbott Laboratories Ltd., London; Tyrothricin from Parke, Davis and Co. Ltd., London; Actidione from The Upjohn Company, Michigan, U.S.A.; Mycostatin from E. R. Squibb and Sons, New York.

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The Bacterial Component of Soybean Root Nodules; Changes in Respiratory Activity, Cell Dry Weight and Nucleic Acid Content with Increasing Nodule Age

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SUMMARY: During the 6- to 7-week active life of soybean root nodules produced by a single strain of *Rhizobium japonicum*, the respiratory activity of the bacteria isolated from the nodules changed according to a definite pattern: a depression occurred 2 weeks after nodules first appeared, followed by a rise, and with a further sharp rise 1 week before nodule breakdown. The initial depression coincided with the onset of the bacteroid condition and the beginning of nitrogen fixation. The limits within which respiration varied depended on host-plant growth. The nitrogen-fixation rate remained constant from 2-3 weeks after nodule appearance until a few days before nodule decay began. The bacteroids isolated from the nodule did not oxidize substrates of the tricarboxylic acid cycle to completion, but the number of mole O_2 consumed/mole substrate did not change during nodule life, and were similar to values obtained for cultures of the free living bacteria. Changes in bacteroid dry weight and nucleic acid content were observed; these were within the limits of dry weight and nucleic acid content of the same bacterial strain grown in liquid culture. The ratio acid soluble nucleotides:nucleic acid for bacteroids was between 0.12 and 0.13 during nitrogen fixation; these values are characteristic of bacteria of the same strain from the mid-logarithmic phase of growth in culture in spite of the fact that bacteroids are non-proliferating cells.

Allen & Allen (1950), Wilson & Burris (1953) and Nutman (1956) have reviewed much of the literature relating to the fixation of atmospheric nitrogen by legume root nodules, and the author (Bergersen, 1957) has summarized the known structural abnormalities of nodules which interfere with their nitrogen-fixing functions. It was shown that all of these abnormalities represented failures in the development of the intimate association between host-plant cell and the symbiotically adapted *Rhizobium*—the so-called 'bacteroids'. The essential role of these bacteroids in the nitrogen fixation accomplished by legume root nodules has been inferred from the results of several workers. For example, Chen & Thornton (1940) showed that differences in the volume and duration of the 'bacterial tissue' accounted for differences in nitrogen fixation in clover, pea and soybean nodules. In contrast, there is no authenticated evidence for nitrogen fixation by *Rhizobium* spp. in culture. It is therefore surprising that there have been relatively few studies of nodule bacteria isolated from nitrogen-fixing nodules.

For the purposes of these studies the term 'bacteroid' will be used irrespective of external morphology for the non-proliferating intracellular forms of *Rhizobium* found in the bacterial tissue of root nodules, in order to distinguish the somewhat unique functions and properties of these forms from those of the

free-living or cultured forms of the bacteria. The following are some of the reports which deal with the properties of bacteroids. Almon (1933) demonstrated the inability of bacteroids from nodules of peas, clover and alfalfa to grow on media which supported growth of the original cultures. Burris & Wilson (1939) and Thorne & Burris (1940) reported studies of the respiration of bacteroids as compared with that of cultures and concluded that the respiration was basically similar in both types of cells. Wilson & Burris (1943) failed to demonstrate hydrogenase in suspensions of bacteroids. Rautanen & Saubert (1955) studied the composition of various soybean and cowpea nodule fractions and showed different distributions of iron, phosphorus, nitrogen and nucleic acids when the bacteroid fraction was compared with cultured bacteria. Cheniae & Evans (1956) found that soybean bacteroids possessed nitrate reductase, whereas the bacteria grown in culture did not unless they were grown in the presence of nitrate. The author (Bergersen, 1955) demonstrated nuclear and cytoplasmic changes during bacteroid formation in subterranean clover nodules.

There is little information in the literature of symbiotic nitrogen fixation about the functions of bacteroids during nodule life; nor have the essential differences between bacteroids and *Rhizobium* organisms in culture been defined, apart from the work quoted above. The present paper reports observations of an experimental system in which bacteroid activity as measured by their respiration in air was examined at intervals during the development and active life of soybean root nodules. At the same time the nitrogen increments of the plants were determined in order to find whether there was any relationship between bacteroid respiratory changes and nitrogen fixation. The non-proliferating nature of the mature bacteroids suggested an examination of their nucleic acid content. The same bacteroid suspensions as used for the respiratory measurements were therefore examined for this purpose. The results from all of these observations were then compared with similar observations made at intervals during growth of the same strain of *Rhizobium* in culture.

METHODS

Plant material. Lincoln strain soybeans (*Glycine max* Merr.) were grown in sterilized soil or in a mixture of sand and vermiculite (1:1, v/v) + nitrogen-free nutrient salts solution (McKnight, 1949), in an air-conditioned glasshouse. The seeds were sterilized with HgCl_2 solution (0.001 %, w/v) and heavily inoculated with the nodule bacteria at sowing and again a few days after the opening of the cotyledons. The plants grown in soil were watered with boiled tap water when necessary and those grown in sand + vermiculite were watered, to maintain the volume of solution originally added, alternately with sterile distilled water or nutrient solution every 2 days. The first experiment was conducted during the summer when the plants grew to 4–5 ft. before flowering; under these conditions successive crops of nodules were produced at about 14-day intervals as the root system enlarged. The time of appearance of the first nodules was recorded and only the oldest sets of nodules were used; these

were easily distinguished by their position and size. The other experiments were conducted during the winter when flowering began 2-3 weeks after germination, and plants stopped making leaf growth once seeds were forming. Under these conditions only one set of nodules was produced on each plant, 15 nodules by the end of the first week from onset of nodulation and a further 8 during the next 2 weeks, after which nodule number remained constant (Fig. 1). Samples of nodules from each harvest were fixed in Flemming's solution, embedded in paraffin wax and microtome sections prepared for examination of detailed nodule development.

Organism. *Rhizobium japonicum* strain CC711 was maintained on yeast-extract mannitol agar. For the examination of bacteria grown *in vitro*, 300 ml. conical flasks containing 100 ml. yeast-extract mannitol broth were inoculated and agitated on a rotary shaker at 20°, a temperature corresponding to the mean soil temperature for the plants grown in the glasshouse. Growth was followed turbidimetrically with an EEL photoelectric nephelometer. Suspensions of rhizobia were concentrated and washed in five changes of buffer.

Bacteroid suspensions. Nodules were harvested at weekly intervals, beginning 1 week after the appearance of the first nodules, and dropped into cold M/15 phosphate buffer (pH 7.0). They were rinsed clean in buffer and then crushed (not ground) with a cold pestle and mortar. The crushed nodules were filtered under slight vacuum through Whatman no. 4 filter-paper to remove nodule debris. The pink suspension was then centrifuged at 300 g for 4 min. in an angle centrifuge to remove coarse starch grains and then at 4500 g for 6-8 min. to sediment the bacteroids. The resulting pink pellet was then washed in four changes of buffer after which 90-96 % of the microscopically visible particles were bacteroids and most of the remainder small starch grains. The concentration of the suspension was then adjusted by turbidity to approximately 10^{10} bacteroids/ml.

Bacterial counts. The numbers of bacteria/ml. suspension were estimated in two ways. (a) 1 ml. of suspension was diluted to 100 ml., 4 drops of 1 % (w/v) Victoria blue 4R added to stain the bacteria, and this suspension counted in a haemocytometer chamber. (b) 1 ml. of suspension was mixed with 1 ml. of a suspension containing an accurately known concentration of formalinized sheep erythrocytes; drops of the mixture were then spread evenly over standard areas of four microscope slides, dried by heat and stained with carbol-fuchsin. The mean ratio of bacteria to erythrocytes in the mixture was determined and from this and the erythrocyte concentration the bacterial numbers were calculated.

Dry-weight determinations. Plant materials were dried at 120° for 8 hr. Bacterial suspensions were dried to constant weight at 100°.

Nitrogen determinations. Dried plant material from the nitrogen-free pot cultures was ground by hand and the quantity of nitrogen/plant determined by the Kjeldahl method. From weekly nitrogen values rates of nitrogen fixation were obtained. Total bacterial-nitrogen was determined according to Umbreit, Burris & Stauffer (1945).

Measurement of oxygen uptake. This was done in a Warburg apparatus at 30° using a shaking rate of 150 oscillations/min. with air as the gas phase. Vessels contained 1.7 ml. of M/15 substrates and 1 ml. of cell suspension in M/15 buffer with 0.2 ml. 20 % (w/v) KOH in the centre well. Where both respiratory rate and mole O₂ used/mole substrate were determined, the vessels contained 0.5 ml. 0.005 M substrate in the sidearms, with 1 ml. of cells in buffer + 1 ml. of buffer in the flask. The first experiments used the Na salts of succinic, fumaric, malic, acetic, citric and pyruvic acids as substrates. In later experiments succinate and citrate were used as being typical of the two types of response being observed. In the control flasks buffer replaced the M/15 substrate and water the 0.005 M substrate. For the purposes of this study oxygen uptakes were expressed in terms of μ l. oxygen/10¹⁰ cells/hr., q_{O_2} (cell) since it was desired to compare cellular activities rather than activity/unit cell constituent (total N or dry weight).

Nucleic acid determinations. The method was based on procedures used by Mitchell & Moyle (1951) and Gale & Folkes (1953). Ten ml. of washed bacterial suspension were extracted at 0° with 2 ml. of 30 % (w/v) trichloroacetic acid for 30 min. The bacteria were then sedimented at about 600 g at 4° and the supernatant fluid containing acid-soluble nucleotides (NT), was collected. The extracted organisms were then washed twice with distilled water and extracted three times for 10 min. at 90° with 2 ml. 5 % (w/v) trichloroacetic acid. These extracts containing nucleic acids (NA) were pooled and made to 10 ml. with distilled water. Further extraction did not increase the NA yield. The NT and NA fractions were slightly opalescent, probably due to traces of lipid in the extracts. They were examined in a Hilger 'Uvispek' spectrophotometer, optical density being measured at 350 m μ to give, by extrapolation, the background density due to light scattering at the nucleic acid absorption peaks, which for the NT was 260 m μ and for the NA 268 m μ . An extinction coefficient of 220 (for 1 % nucleic acid in a 1 cm. light path absorption cell) was used for the NT fraction and 280 for the NA (Malmgren & Heden, 1947; Gale & Folkes, 1953). All spectrophotometric measurements were made against blanks containing the same reagents as the extracts and all estimations were done in triplicate.

RESULTS

Nodule development

The structural development of the nodules used in these experiments merits emphasis. Microscopic examination of samples of nodules from each harvest revealed two important features.

(a) The bacterial tissue in individual nodules was uniform (in contrast to that of elongating nodules from other types of legumes). This was because nodule development proceeded in well-defined stages: first a small locus of infected cells and the surrounding root cortex cells multiplied to give a ball of small host cells each containing a few bacterial rods and surrounded by the developing cortex, vascular system and endodermis of the nodule. Next the bacteria multiplied greatly and the host cells containing them enlarged; this

was complete in most nodules by the second harvest, 2 weeks from the appearance of the first nodules. The central cells of the nodules and the bacteria within them then increased in size but not in number until 4 weeks from nodule appearance; the nodule structure then remained static until 6–7 weeks when the nodule contents began to degenerate. Thus the first 2 weeks of nodule development was the total period of bacterial proliferation, until decay began. These microscopic findings agree with the nodule weight data of Fig. 1.

(b) The nodule samples were themselves fairly uniform due to the selection of nodules of similar age or due to the production of only one set of nodules on each plant.

From this information it seems logical to assume that the activities and constitution of the pooled bacterial suspensions were representative of the individual bacteria within any nodule cell at the particular stage of development at which the nodules were harvested, except in the case of harvest 2 when the suspension largely consisted of bacteroids, but with a few proliferating bacteria from the nodules in which cell division was still occurring.

Nitrogen fixation

From the total plant nitrogen of nodulated plants grown in nitrogen-free culture, under conditions where only one set of nodules was produced, it was found that plant-total nitrogen increased at a constant rate from about $2\frac{1}{2}$ to

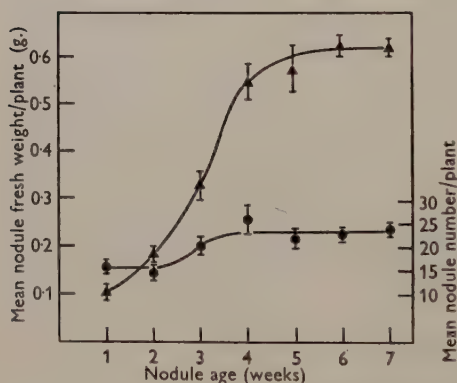


Fig. 1

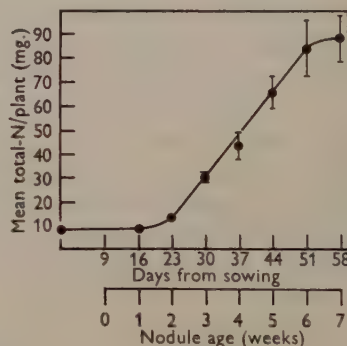


Fig. 2

Fig. 1. Nodule growth of plants growing in N-free culture in winter. The vertical lines indicate the magnitude of the standard errors of the means. ●—●=mean nodule number/plant; ▲—▲=mean nodule fresh weight/plant.

Fig. 2. Total nitrogen of nodulated plants grown in N-free culture in winter. The vertical lines indicate the magnitude of the standard error of the means.

6 weeks after the beginning of nodulation. Nodule decay had begun by 7 weeks and fixation had ceased (Fig. 2). From germination the foliage of the plants became increasingly yellow until 15 days after the beginning of nodulation, when the plants became bright green overnight.

Bacteroid respiration

The first experiments with bacteria from nodules on summer-grown plants revealed that the values of q_{O_2} varied with the age of the nodules. This variation was greater with succinate and fumarate than with the other substrates with which respiration closely followed changes in endogenous activity (Fig. 3*a*). There was a sharp decrease in respiration with all substrates 1 week after the onset of nodulation, followed by a rise, then a steady period followed by a sudden and very significant rise when the nodules were about 7 weeks old. This general pattern was again obtained with nodules grown during the winter in nitrogen-free culture or in soil. However, the values of respiration within which the above variations occurred differed between nodules from summer and winter-grown plants and between plants grown in nitrogen-free culture and in soil (Fig. 3*a-c*).

None of the tricarboxylic acid cycle intermediates tested as substrates with whole bacteroids caused the theoretical oxygen uptake for complete oxidation (Table 1*a*). However, the molar uptakes of oxygen by the bacteroids differed

Table 1. *Incomplete oxidation of tricarboxylic acid cycle intermediates by bacteria from culture and bacteroids from nodules*

Warburg vessels contained bacterial suspensions in M/15 phosphate buffer with 0.5 ml. of the 0.005 M substrates as Na salts in the sidearms. All measurements were made at 30° with a shaking rate of 150 oscillations/min. CO₂ was absorbed by 0.2 ml. 20% (w/v) KOH on a filter-paper wick in the centre well. The substrates were added by tipping after 20 min. equilibration followed by measurement of endogenous activity for half an hour. O₂ uptake/mole substrate was calculated from differences between endogenous uptake and that due to added substrate when the rates of uptake in the flasks with added substrate returned to the endogenous rate.

(a)

Substrate	Oxygen uptake (mole O ₂ /mole substrate)		
	Bacteria from 10-day-old culture	Bacteroids from 5-week-old nodules	Theoretical to completion
Succinate	0.8	1.0*	3.5
Fumarate	0.4	1.0	3.0
Malate	0.3	0.3	3.0
Pyruvate	0.0	0.3	2.5
Acetate	0.5	0.8	2.0
Citrate	0.8	1.0	4.5

(b)

Bacteroids from nodules aged (weeks)	Oxygen uptake— mole O ₂ /mole succinate
1	1.0
2	1.1
3	0.9
4	0.9
5	1.1*
6	1.2
7	1.1

* Different experiments.

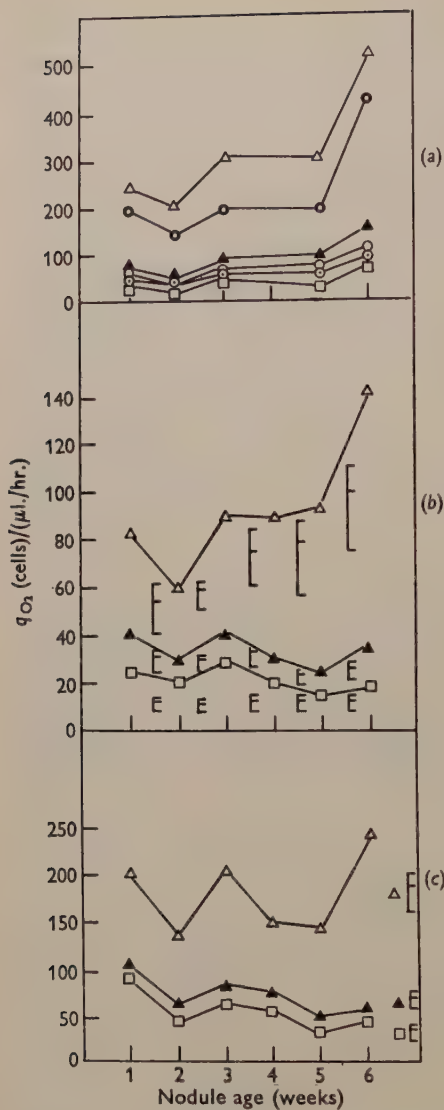


Fig. 3

Fig. 3. Changes in bacteroid respiration with age of nodules. Plants grown in: (a) soil in summer; (b) N-free culture in winter; (c) soil in winter. Δ - Δ =succinate; \circ - \circ =fumarate; \blacktriangle - \blacktriangle =citrate; \circ - \circ =acetate and malate; \circ - \circ =pyruvate; \square - \square =endogenous.

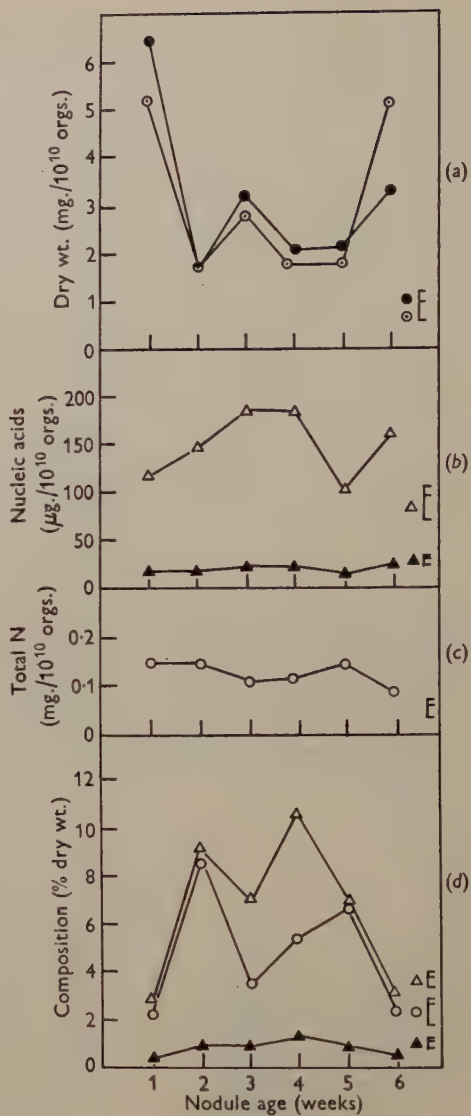


Fig. 4

Fig. 4. Changes in bacteroid composition with age of nodules. (a) Changes in organism dry weight: \circ - \circ =plants grown in soil in winter; \bullet - \bullet =plants grown in N-free culture in winter. (b) Changes in organism nucleic acid; Δ - Δ =NA; \blacktriangle - \blacktriangle =NT. (c) Changes in cell-N total: \circ - \circ . (d) Changes in percentage composition: symbols as in (b) and (c). Differences for significance between successive points are shown thus— $\left[\begin{array}{l} 1\% \\ 5\% \end{array} \right]$ where these are of similar magnitude, only the greatest is shown.

only slightly from those by the bacteria grown *in vitro*. The molar uptakes of oxygen for succinate throughout nodule life remained fairly constant (Table 1*b*). It therefore seems that there is little or no change in pathways of substrate oxidation as between bacterium and bacteroid.

Respiration of bacteria grown *in vitro*

Respiratory rates (q_{O_2} cell) for cultures of various ages are illustrated in Fig. 5*e* which shows that respiration was highest during active growth (Fig. 5*a*).

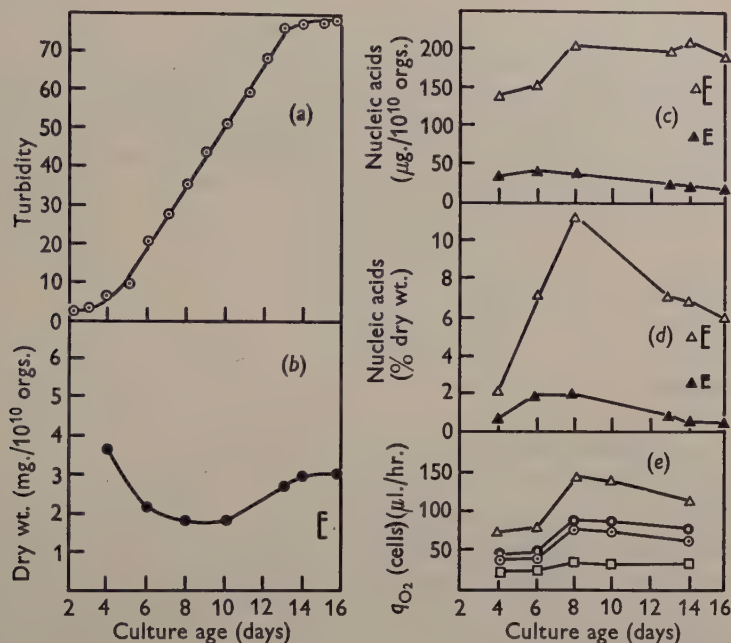


Fig. 5. Organism dry weight, nucleic acid content, and respiration of organisms from liquid culture at 20°, (a) growth as measured by turbidity; (b) organism dry weight; (c) organism nucleic acid, etc.; Δ - Δ =NA; \blacktriangle - \blacktriangle =NT. (d) % (w/w) nucleic acid: symbols as in (c). (e) Oxygen intake: Δ - Δ =succinate; \bullet - \bullet =fumarate; \circ - \circ =malate; \square - \square =endogenous. Differences for significance between successive points are shown

thus — $\begin{matrix} 1\% \\ 5\% \end{matrix}$; where these are of similar magnitude only the greatest is shown.

Changes in bacteroid constitution

From the values for dry weight and bacterial count for suspensions from two experiments, the values of dry weight/10¹⁰ organisms were calculated. Figure 4*a* shows the patterns of change.

Changes in nucleic acid, acid-soluble nucleotide and total-N content for bacteroids from plants grown in soil are illustrated in Fig. 4*b* and *c*. Figure 4*c* shows that the changes in dry weight/10¹⁰ organisms must be due to non-nitrogenous constituents since total-N is almost constant. Nucleic acid would appear to play no part in the dry-weight changes from 1 to 2 weeks.

The dry-weight increase at 2-3 weeks has a nucleic acid component as has the increase at 5-6 weeks, but these are only about 5% of the corresponding dry weight changes.

When nucleic acid, acid-soluble nucleotides and total-N are expressed as % dry wt. organisms it will be seen (Fig. 4*d*) that the pattern of change with time is almost a mirror image of the dry-weight pattern (Fig. 4*a*). This emphasizes that the magnitude of the dry-weight change is far greater than the change in any of the constituents examined.

Table 2. *The ratio acid-soluble nucleotide:nucleic acid for bacteria from in vitro culture and bacteroids from nodules*

Acid-soluble nucleotide (NT) was assayed by measurement of optical density at 260 $m\mu$ of a 30% (w/v) trichloroacetic acid extract of the organisms made at 0° for 30 min. Nucleic acid (NA) was assayed by measurement of optical density at 268 $m\mu$ of a pooled extract made by extracting organisms with 5% (w/v) trichloroacetic acid three times at 90° for 10 min. *In vitro* cultured organisms were *Rhizobium japonicum* strain CC711 grown in yeast + mannitol broth at 20°. Bacteroids came from nodules of Lincoln soy bean plants grown in soil.

Bacteria grown <i>in vitro</i>							
Age of culture (days)	6	8	13	14	16		
Ratio, NT:NA	0.27	0.18	0.12	0.10	0.09		
Bacteroids							
Nodule age (weeks)	1	2	3	4	5	6	7
Ratio, NT:NA	0.15	0.12	0.13	0.13	0.13	0.18	0.25

Changes in the composition of bacteria from culture

The growth at 20° of the strain of *Rhizobium* used is illustrated by the turbidity/time curve of Fig. 5*a*, and the changes of dry weight and nucleic acid content/ 10^{10} organisms at various stages of growth are shown in Fig. 5*b-d*. While nucleic acid (% dry wt.) varied inversely with dry weight, the nucleic acid/organism remained constant at 200 $\mu\text{g.}/10^{10}$ organisms during the most active stage of growth.

DISCUSSION

Patterns of change of respiration, cell dry weight, acid-soluble nucleotides and nucleic acid have been established for the developing bacterial component of soybean root nodules. In the experimental system used in this work there was a decrease in the respiratory activity of the bacteria isolated from the nodules before the beginning of nitrogen fixation, which began when the nodules were approximately 2 weeks old. From the microscopic observations this corresponds to the cessation of bacterial proliferation within the host cells, i.e. the beginning of the bacteroid condition. Further changes in respiratory rates of the bacteroids during the functional life of the nodules bore some relationship to changes in the rates of nitrogen fixation. When the weekly plant nitrogen increments from Fig. 2 are compared with the q_{O_2} of the bacteroids from the nodules on those plants (Fig. 3*b*) it will be seen that the increase in N-increment from 2 to 3 weeks corresponds with an increase in respiration: the increments then remained fairly steady from 3 to 5 weeks when respiration changed only

a little. The sharp increase in respiration which preceded nodule breakdown, however, occurred while the steady rate of nitrogen fixation was continuing. From this it seems that the changes in respiration which occur in the bacteroids are not always directly concerned with the nitrogen-fixing functions of the tissue. This contrasts with nitrogen fixation in *Azotobacter* where there is a close relationship between amount of substrate oxidized by the bacteria and amount of N_2 fixed (Wilson & Burris, 1943), and hence between respiratory rate and N_2 -fixation rate.

The general pattern of respiratory change of the bacteroids during the nodules' active life was reproduced on three occasions, although the limits within which the changes occurred in any one experiment seemed to be related to the size of the plants from which the nodules were harvested. The summer-grown plants were the largest (20–60 g. top fresh weight) with the highest q_{O_2} for the bacteroids, while with winter-grown plants, those grown in soil benefited from soil nitrogen and were larger (5–25 g. top fresh weight) and had higher q_{O_2} values for the bacteroids than those grown in N-free culture (2–10 g. top fresh weight). The bacteroid respiration followed a similar pattern to changes in bacteroid dry weight. In contrast the cultured bacteria had the highest q_{O_2} values when they were growing most rapidly and when the organism dry weights was lowest. The failure of this strain of *Rhizobium japonicum* to completely oxidize substrates of the Krebs cycle suggests that this cycle may not operate in these organisms. If it is blocked there may be accumulation of one or more of the acids concerned in the preceding steps or these acids may be diverted to other processes. If the Krebs cycle does operate in these bacteria it would seem that the substrates supplied are oxidatively assimilated, a portion being completely oxidized to provide energy for the conversion of the remainder to lipid or carbohydrate reserve materials. These possibilities are at present being examined and will be the subject of a future communication.

Mitchell & Moyle (1951) and Gale & Folkes (1953) found that growth and protein synthesis in *Staphylococcus* were related to % (w/w) ribonucleic acid (RNA), growth being most rapid when this was at a maximum. They also concluded that RNA was synthesized from the acid-soluble nucleotides which attained maximum concentration before the RNA. In *Rhizobium japonicum* this relationship was also found to hold for growing cultures, % (w/w) nucleic acid (NA) being greatest during the logarithmic phase of growth, and acid-soluble nucleotide (NT) attaining its maximum concentration 2 days before nucleic acid reached its maximum. The work reported here has shown the desirability of relating % (w/w) nucleic acid to cellular nucleic acid content, since increases in the former value may be artefacts resulting from changes in organism dry weight and thus not mean that nucleic acid synthesis has occurred. The NT/NA ratio of 0.12 during the active growth of this strain of *Rhizobium japonicum* is comparable with the Mitchell & Moyle (1951) value of 0.1 for the NT/(RNA + DNA) ratio of actively growing cells. In the bacteroids, however, both the NA content/ 10^{10} organisms and the % NA content fell within the same limits as with growing cells at times during the experiment, although the NT values of the bacteroids were always lower than those of

cultured bacteria. As has been shown, there was a change over from the proliferating bacterial form to the bacteroid form about 2 weeks after nodule appearance. During this period % (w/w) NA increased to a value characteristic of actively proliferating cultures. This was largely due to a decrease in organism dry weight, NA/organism increasing only slightly. It seems unlikely therefore that the cessation of bacterial division within the nodule was caused by lowered nucleic acid content as suggested by Rautanen & Saubert (1955). These authors found 2.98 % (w/w) NA in bacteroids from soybean nodules and 8.25 % (w/w) in organisms grown *in vitro* but found smaller differences between bacteroids and organisms grown *in vitro* with cowpea nodule bacteria. Figs. 4d and 5d illustrate the danger of making these types of comparisons unless ages of nodule and culture are specified since both NA/organism cell and % NA are dynamic quantities. Rautanen & Saubert's (1955) figures, on the other hand, do not disagree with the figures presented in this paper when the culture was in early or late logarithmic phase and the nodules mainly 5-6 weeks old. It is more probable, however, that the figure of 2.98 % (w/w) is an average for nodules of a variety of ages.

Although patterns of change with time of the various features examined in bacteroids differed, as one would expect, from the patterns of cultured bacteria, the general ranges of values obtained were similar in both types of cells. Other characteristics must therefore be examined in order to define differences between bacteroids and the organisms of *Rhizobium* cultivated *in vitro*.

The author wishes to record his thanks to Mr G. A. McIntyre for the analyses of significance and to Mrs M. Stiller for technical assistance.

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The Keeping Quality of Freeze-Dried *Brucella abortus* Strain 19 Vaccine

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SUMMARY: Concentrated *Brucella abortus* strain 19 vaccine is shown to deteriorate in time, even at low temperatures (4-7°). The loss of viability of vaccine sealed in pure nitrogen can be expressed as a coefficient. The value of this coefficient may be derived from the formula of a straight line fitting the means of the logarithms of the viability counts made 1 week to 6 months after freeze-drying.

Conventional liquid *Brucella* vaccine has poor keeping qualities because it consists of *Brucella abortus* strain 19 organisms which have to be fully viable to confer immunity on the inoculated animal. In the saline suspension of 60,000,000,000 organisms/5 ml. dose, which is the usual concentration of liquid vaccine, the viability of the organisms is progressively destroyed by light, heat and agitation during transport. The vaccine keeps fairly well for several weeks at 4° and is best stored at this temperature, but transport at 4° is rarely practicable, and oxidative processes destroy viability as soon as the temperature is raised. No chemical preservative can be used in this vaccine, as no toxoid or sporulating phase is involved, the immunizing quality being solely dependent on the high concentration of living bacteria.

Verwey & Scheidy (1944) used the freeze-drying technique to maintain the viability of *Brucella abortus* strain 19 vaccine in an attempt to eliminate losses due to agitation and oxidation during transport and storage. Freeze-dried vaccine is, however, still susceptible to detrimental influences of heat and light (Naylor & Smith, 1946). Alexander & van Drimmelen (1956) freeze-dried this vaccine in highly concentrated form for economical distribution, the ampoules being sealed in purified nitrogen. Tests have proved this vaccine to have exceptional keeping qualities at room temperature (G. N. van Waveren *et al.* personal communication), but it is, of course, not superior to other freeze-dried *Brucella* vaccines when those are held at 4°.

All vaccines can be stored at this temperature for long periods, the loss of viability probably amounting to only a fraction of a logarithm of the viable content. However, some uncertainty was found to exist concerning the exact degree of deterioration in viability taking place under the most favourable storage conditions. As it was found desirable to stockpile *Brucella* vaccine at distribution points in South Africa as far as a thousand miles by rail from the producing laboratory, an accurate prediction of the viable count of stored

freeze-dried vaccine became a necessity. The purpose of this report is to describe a method whereby the rate of loss of viability of different types of vaccine can be estimated.

METHODS

The vaccine used in this work was described previously by van Drimmelen (1956). It consisted of sodium carboxymethylcellulose-sedimented, concentrated *Brucella abortus* S. 19, freeze-dried in 4 ml. amounts in 10 ml. ampoules and sealed in nitrogen. The number of micro-organisms surviving in freeze-dried material depends on the species and concentration of organisms, the suspending medium, and the method of freeze-drying and sealing of the containers. Where storage methods and freeze-drying techniques are constant, the production methods should be the only factors which influence the keeping quality of the product. Different types of vaccine were, therefore, produced, varying only in respect of viable count and freeze-drying mixtures, as follows:

Vaccine 1. Concentration of 160 ml. packed organisms/l. containing 0.5 % (w/v) ascorbic acid + 0.25 % (w/v) thiourea (antioxidant).

Vaccine 2. 320 ml. packed organisms/l. containing 0.5 % (w/v) ascorbic acid + 0.25 % (w/v) thiourea.

Vaccine 3. 160 ml. packed organisms/l. containing 1.8 % (w/v) ascorbic acid + 2.8 % (w/v) thiourea.

Vaccine 4. 320 ml. packed organisms/l. containing 1.8 % (w/v) ascorbic acid + 2.8 % thiourea.

Counts were made of a number of viable organisms/ampoule from a representative number of batches of each vaccine, by the method of Miles & Misra (1938) with minor modifications. These counts were made at selected time intervals during storage after freeze-drying, namely, immediately after drying and then after 1, 2, 3 weeks and 1, 3 and 6 months. The resultant figures were grouped for analysis in units of 10^9 viable organisms/ampoule, i.e. the figure 2250 represents a count of 2250×10^9 viable organisms/ampoule.

To examine the distribution of viable counts at any particular time interval, a careful study was made of cases where more than thirty counts were available. As skew distributions were found on all occasions the cumulative frequencies were plotted on logarithmic probability paper and this resulted in approximately straight lines (Fig. 1), which indicated that the distribution was approximately log. normal. The observed counts are mean values multiplied by dilution factors which are not always constant; therefore, the distributions could not be considered as being Poisson distributions, which are known to occur frequently in such counting experiments.

χ^2 tests were used to test the goodness of fit with the 'log. normal distribution' and gave satisfactory results. It was thus decided to use the logarithms of the observed counts (in thousand millions) for the selected periods, and to take them as being distributed normally.

The mean value of the logarithms of the counts was calculated for each period after freeze-drying, to find the trend in the deterioration of the

logarithms. The 'standard deviations' and 'standard errors' were calculated independently for each of the distributions at the selected periods.

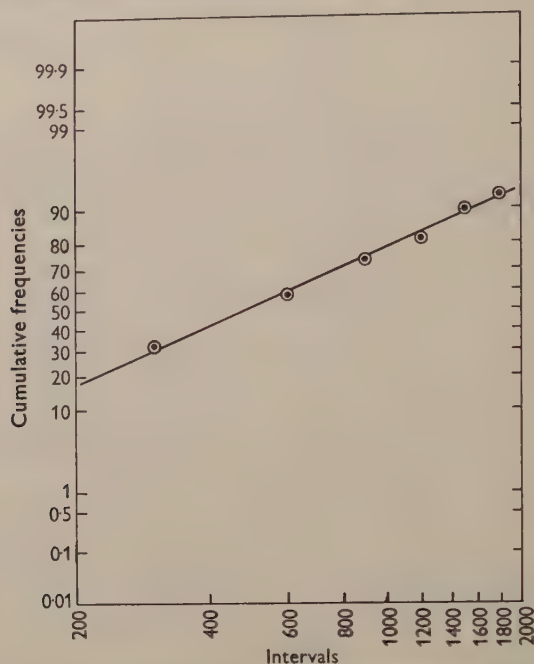


Fig. 1. Example showing that the relative cumulative frequencies of the viable counts when plotted on logarithmic probability paper give approximately a straight line. This indicates that the distribution at any particular time interval is approximately a log normal distribution.

RESULTS

The results are shown in Fig. 2 with the time periods, x on the horizontal axis and the mean logarithms of the counts, y on the vertical axis. The equations of the straight lines found to run through these mean values, obtained by the method of least squares, were as follows:

A. Vaccine 1 $y = 2.682 - 0.012x$.

Vaccine 2 $y = 2.675 - 0.018x$.

B. Vaccine 3 $y = 3.292 - 0.017x$.

Vaccine 4 $y = 3.233 - 0.017x$.

The results are illustrated in Fig. 2. Calculation of the standard error and standard deviation gives indications that the curves provide a method of characterizing the quality of the given types of *Brucella* vaccine by means of a coefficient derived from equations (1) to (4) above. The relations are found to be linear, which means that the rate of change of y with x remains constant, and is equal to the coefficient of x . The values of the coefficient of x were: vaccine 1, 0.012; vaccine 2, 0.018; vaccine 3, 0.017; vaccine 4, 0.017. These coefficients show the average decrease in the mean of the logarithms of the counts per week.

In Fig. 2 two limits are shown on each side of the mean for each time period. The outer limits are, respectively, at the points given by the mean + two standard deviations, and the mean - two standard deviations. These limits are such that only one out of twenty of the logarithms of the counts can be expected to fall outside them. For all practical purposes, therefore, the logarithms of all the counts at a particular time period may be taken as being between these two limits. The inner limits are respectively at points given by the mean + two standard errors and the mean - two standard errors. These are limits for the calculated mean values and such that only one out of twenty mean values from such samples can be expected to fall outside them.

As is indicated on the graphs on Fig. 2 the estimated mean values of the logarithms are never further than 2 standard errors from the straight lines. Since the standard deviations of the distributions of the logarithms were nearly always found to be smaller or equal to 0.4, it is suggested that for any mean value estimated from the graphs at any time period x_1 the maximum standard error may be taken as approximately equal to $0.4/\sqrt{n}$. This should be interpreted as follows. If at this time period x_1 n counts were available, then the mean of the logarithms of the counts will not differ by more than $2 \times 0.4/\sqrt{n}$ from the mean value on the graph. These values are specifically applicable to sodium-carboxy-methyl-cellulose concentrated vaccine.

DISCUSSION

Statistical analysis of the results shows that the rate of loss of viability of any particular type of freeze-dried *Brucella* vaccine can be characterized by a constant which may be termed the coefficient of viability loss. The discovery of this constant is believed to be of practical value in organizing the distribution of vaccine in widespread areas, by making it possible to predict the viable count of vaccine stored at 4° at distribution centres. Vaccine from the distribution centres could then be correctly labelled, as regards dosage, for periods of several months after leaving Onderstepoort. It is also a welcome discovery for use in studying the methods of production and freeze-drying, and should contribute to the replacement of empirical methods of freeze-drying by rational procedures.

It is clear from the values of the coefficient of x that at the lower concentration of anti-oxidant (0.5 % ascorbic acid + 0.25 % thiourea), the rate of viability loss was greater in the more concentrated vaccine (vaccine 2), i.e. 0.018, as opposed to 0.012 in the less concentrated vaccine (vaccine 1), whereas at the higher concentration of anti-oxidant the rate of viability loss was the same in both concentrations of vaccine, i.e. 0.017 (vaccines 3 and 4). At the higher concentration of vaccine (320 ml. packed organisms/l.) the rate of viability loss was greater in the vaccine containing the lower amount of anti-oxidant (vaccine 2), but at the lower concentration of vaccine (160 ml. packed organisms/l.), the rate of viability loss was greater in the vaccine containing the higher amount of anti-oxidant (vaccine 3). This indicates that excess anti-oxidant and insufficient anti-oxidant both adversely affected the

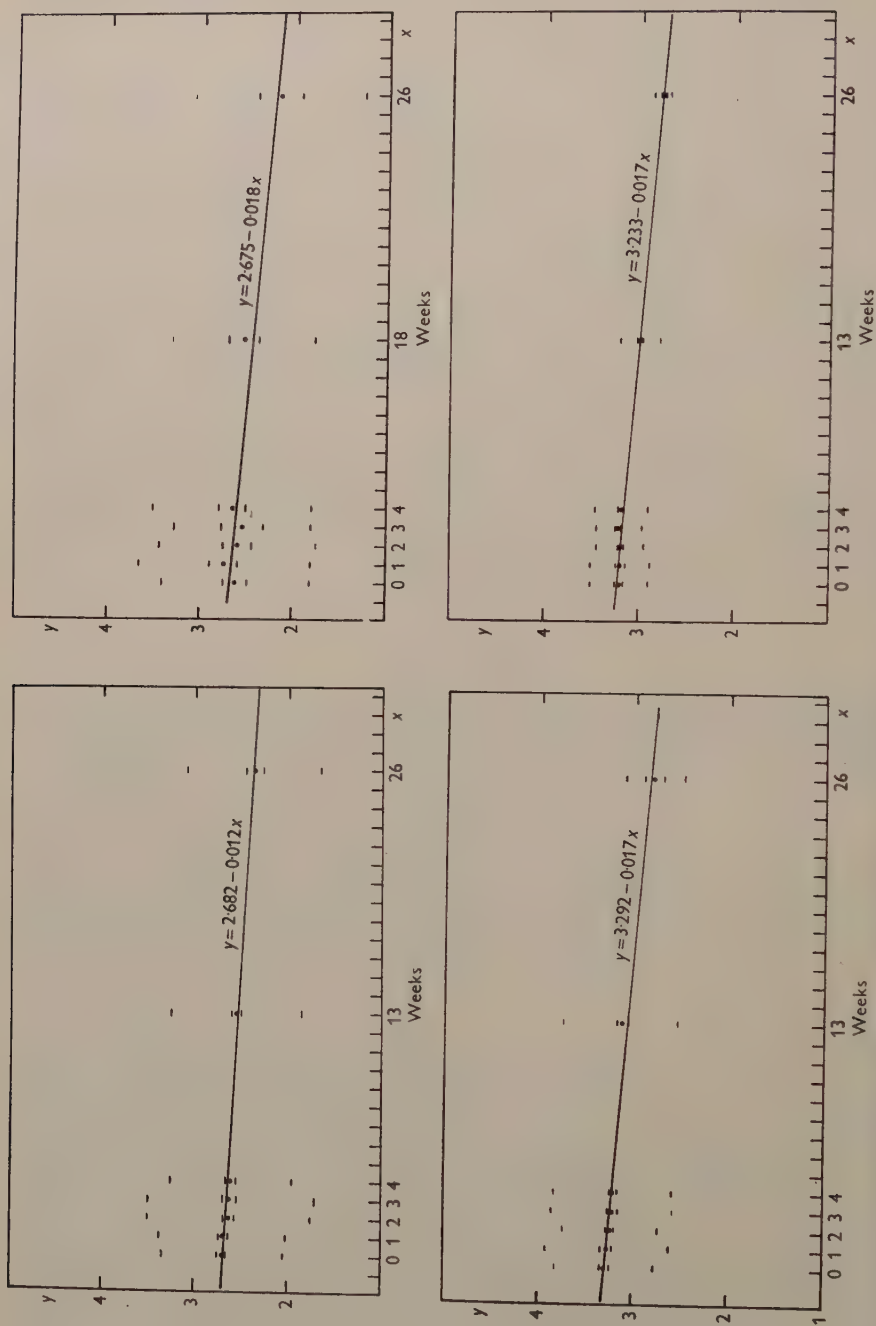


Fig. 2. Characterization of four Brucella vaccines by graphs of the mean logarithms of the viable counts, showing a linear decline described by the given equations.

viability of the vaccine during storage, and shows that for maximum stability the anti-oxidant incorporated in the freeze-dried vaccine should be balanced with the concentration of viable organisms present.

It is known from experience that more concentrated vaccine requires higher anti-oxidant protection during freeze-drying than less concentrated vaccine, and evidence is available that excess of anti-oxidant during freeze-drying also is detrimental to the vaccine. This aspect is being analysed at present.

The present data show that an increase of anti-oxidant in freeze-drying mixtures for vaccines of 160 ml. packed organisms/l., although increasing the survival rate immediately after freeze-drying from a mean log. viable count of 2.682 to a mean of 3.292 (graphs 1 and 3), also results in an increased rate of deterioration during storage (coefficients of viability loss 0.012 and 0.017). For vaccines of 320 ml. packed organisms/l. an increase in anti-oxidant causes both an increase in immediate survival (values 2.657 and 3.233 graphs 2 and 4), and an increased stability during storage (coefficients 0.018 and 0.017). Based on data of this nature a system of distributing concentrated *Brucella* vaccine in bulk in refrigerated containers to advance centres of storage and distribution can be arranged. The vaccine can be labelled at the time of final issue, and the dosage calculated, in relation to the known rate of deterioration, by applying the correct formula for the type of vaccine used.

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An Aromatic Intermediate in the Bacterial Oxidation of Quinic Acid

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SUMMARY: Strains of *Pseudomonas* and *Achromobacter* spp. isolated from soil, utilized quinic acid as a sole source of carbon. Protocatechuic acid was produced as an intermediate in this oxidation. Protocatechuic acid was also produced in the course of oxidation of *p*-hydroxybenzoic acid by these organisms. Sequential induction experiments indicate that *p*-hydroxybenzoic acid is not an intermediate in quinic acid oxidation by the organisms used.

Aromatic compounds are produced by micro-organisms in the synthesis of the aromatic constituents of their own cell substance. Apart from these anabolic syntheses certain micro-organisms produce aromatic metabolic products in the course of oxidative assimilation of non-aromatic materials. One such product is 6-hydroxy-2-methyl-benzoic acid, produced by *Penicillium griseofulvum* Dierckx from acetate (Birch, Massy-Westropp & Moye, 1955). Many of the anthraquinone pigments produced by moulds are in this category. Emmerling & Abderhalden (1903) showed that quinic acid (1:3:4:5-tetrahydroxycyclohexanecarboxylic acid) was attacked by an organism named *Micrococcus chinicus*, giving rise to protocatechuic acid. Butkewitsch (1924) reported that certain moulds produced protocatechuic acid and catechol from quinic acid; the catechol was assumed to have arisen from protocatechuic acid. This, however, is not in keeping with current concepts of protocatechuic acid oxidation (Umbreit, 1952; Evans, 1956).

The present work was begun to determine whether protocatechuic acid produced from quinic acid represents an intermediate in quinic acid oxidation or a metabolic end product, to determine the quantitative aspects of the conversion, and if possible to determine other aromatic intermediates.

METHODS

Pure cultures of quinic acid-oxidizing bacteria and fungi were isolated from soil by using an enrichment technique. Enrichments and fermentations were carried out, and stock cultures maintained, on a medium of the following composition (g.): quinic acid, 10.00; NH_4NO_3 , 4.00; MgSO_4 , 0.50; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.10; $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.05; K_2HPO_4 , 1.0; CaCO_3 , 2.0; in distilled water, 1 l. The medium was prepared as two double-strength solutions, one containing only the K_2HPO_4 . The quinic acid was dissolved in the phosphate solution and the pH value adjusted to 7.4 with 10% (w/v) KOH. The two solutions were then mixed and sterilized at 121° for 20 min. When a solid medium was

desired agar was added to 1.5 % (w/v). The final pH value of the medium was 7.2 ± 0.05 .

Organisms for manometric experiments were grown on mineral salts agar containing 0.3 % (w/v) of the appropriate carbon source. Six Kolle flasks, each containing 50 ml. of agar medium, were inoculated with 1.0 ml. each of a 24 hr. liquid culture of the organism used. After 24 hr. of incubation at room temperature, the growth in each flask was washed off with 20 ml. M/25 phosphate buffer (pH 7.38). An equal volume of buffer was added to the pooled washings, the organisms deposited by centrifugation at 5°, and washed twice in buffer and resuspended in 15 ml. buffer. Before use, dry-weight determinations of organisms in the suspensions were made and the suspensions adjusted to contain 1.0 mg. bacterial-N/ml.

The Warburg apparatus was used in the conventional manner to measure oxygen uptake (Umbreit, Burris & Stauffer, 1957). Each vessel was made up with 1.0 ml. buffer containing 3 μ mole of substrate in the sidearm, 0.15 ml. of 20 % (w/v) KOH and a 2 cm. square of Whatman No. 42 filter-paper in the centre well, and 1.0 ml. of cell suspension and enough buffer to bring the volume to 3.0 ml. in the flask. All manometric experiments were run at 30°.

Analyses of ultraviolet (u.v.) absorption were made in a Cary Model 11 ultraviolet spectrophotometer, with buffer of 95 % (v/v) ethanol in water as solvent. Quantitative determination of protocatechuic acid was made by measurement of absorption at 290 m μ , or 293 m μ if the sample was acidified to reduce scattering (Fig. 1). Concentrations were calculated from a standard curve prepared by plotting D 293 against concentration of protocatechuic acid in the range 0.005–0.05 mg./ml., or by baseline calculations made by the method of Wright (1941). A check on the identity of the compound being determined was made with the 257 and 293 m μ readings which bear the constant relationship $D\ 257/D\ 293 = 1.95 \pm 0.05$ for protocatechuic acid. Infra-red analyses were made in a Perkin-Elmer Model 21 double-beam infrared spectrophotometer. Samples were prepared for analysis as potassium bromide pellets.

RESULTS

Isolation of cultures

Flasks (1 l. Erlenmeyer) containing 200 ml. of medium were inoculated for enrichment of the desired organisms with 1 g. each of a garden soil. After 48 hr. of incubation at room temperature, a heavy growth of micro-organisms, predominantly fungal, was noted in all flasks. Subcultures were made by streaking plates of quinic acid agar with a loopful of liquid culture. Selection and subculture on the same medium yielded over 40 pure cultures capable of using quinic acid as a sole carbon source. About 2/5 of the cultures were moulds of the genera *Penicillium* and *Aspergillus*. The bacteria isolated were Gram-negative rods, except for a few Gram-variable irregularly staining forms tentatively identified as *Corynebacterium* spp. The bacteria used were of three types, and a representative culture of each type was used in these studies.

The characteristics of the representative cultures chosen for these studies are summarized in Table 1.

Cultures A-5 and D-19 are members of the genus *Pseudomonas*. A-5 closely resembles the type species *P. aeruginosa*, exuding a marked odour of trimethylamine, and is so designated; D-19 is not identical with any of the species reported in *Bergey's Manual* (1948). Culture E-20 is apparently a member of the genus *Achromobacter*, and resembles the aromatic hydrocarbon-attacking *A. cycloclastes*.

Table 1. *Morphological and biochemical characteristics of bacteria capable of using quinic acid as sole carbon and energy source*

Characteristic	Isolate		
	A 5	D19	E20
Growth on quinate liquid medium	Green top, deep purple bottom	Purple throughout tube	Pale faintly fluorescent bluish colour
Gram reaction	Gram-negative very small rods	Gram-negative small rods	Gram-negative rods
Colonies on nutrient agar	3 mm., irregular, smooth centre, radiate edge, convex, translucent, agar pinkish	Small, irregular, rough, butyrous, convex, iridescent	Smooth, round, convex, white, opaque
Nutrient agar slope	Spreading, smooth, radiate edge, glistening, agar pink	Spreading, rough, iridescent	Thick, smooth, white, glistening, filiform
Nutrient broth	Moderate turbidity, pellicle	Moderate turbidity, pellicle	Heavy turbidity, pellicle
Motility	+	+	+
Gelatin liquefaction	+	—	—
Nitrate reduction	→NO ₂	—	→N ₂
Litmus milk	Peptonization, slow reduction, blue-grey pellicle	No action	No action
Glucose, sucrose, lactose broth	No acid or gas	No acid or gas	No acid or gas
Asparagine broth	Pale-green soluble pigment	Pale yellowish green soluble pigment	No pigment

Protocatechuic acid formation from quinic acid

Each culture produced a characteristic coloration in deep tubes of quinic acid liquid medium (Table 1). Ferric chloride tests (Soloway & Rosen, 1958) with an authentic sample of protocatechuic acid gave a deep green colour. Tests of the reaction of ferrous chloride with protocatechuic acid showed that the acid gave a purple colour with ferrous ion; the test medium in the tubes was prepared with ferrous chloride. The characteristic two-colour appearance of the tubes of culture A-5 could be duplicated in a solution of protocatechuic acid and ferrous chloride, the top of the tube becoming green on standing owing to oxidation of ferrous ion to the ferric state. Samples taken from

48 hr. liquid medium tube cultures gave a green colour with ferric chloride, regardless of purple coloration in the tube.

In shaken flask experiments, 400 ml. portions of quinic acid liquid medium in 2800 ml. Fernbach flasks were inoculated with 25 ml. of 48 hr. liquid cultures of the organisms tested. After 24–48 hr. of shaking at room temperature the culture fluid took on a violet to purple colour, which persisted for an additional 18–24 hr. period. Samples of culture fluid taken during the period of colour formation gave green a colour with ferric chloride and had a u.v. absorption spectrum identical with that of an authentic sample of protocatechuic acid. Some scattering occasionally interfered with the spectrum but was dissipated by the addition of a drop of conc. HCl diluted with an equal volume of water to the absorption cell (Fig. 1). Quinic acid gave no interfering absorption in the ultraviolet region.

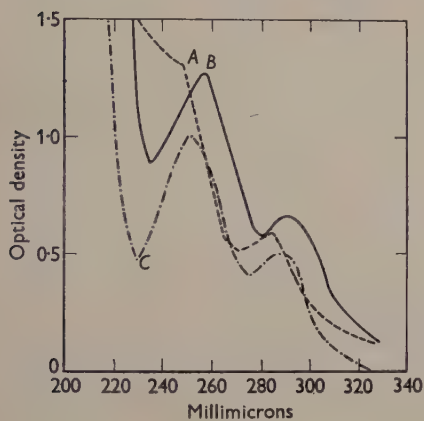


Fig. 1

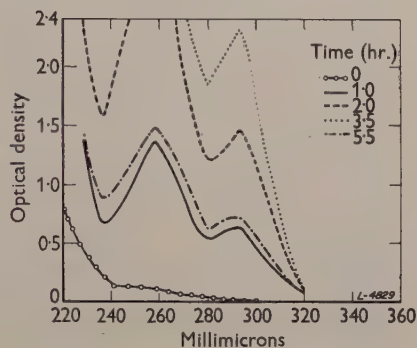


Fig. 2

Fig. 1. Ultraviolet absorption spectrum of protocatechuic acid in treated and untreated quinic acid medium cultures.

Fig. 2. Ultraviolet absorption spectra of a washed suspension (organism A-5) producing protocatechuic acid from quinic acid.

The production of protocatechuic acid by a washed suspension of organisms was determined as follows. Quinic acid (250 mg.) was dissolved in 25 ml. of *m*/50 phosphate buffer (pH 7.38), in a 500 ml. Erlenmeyer flask and neutralized with 4% (w/v) aqueous KOH. One ml. of a solution containing 2.5 mg. $\text{MgSO}_4/\text{ml.}$, and 10 ml. of a washed suspension of organisms equivalent to 1.0 mg. bacterial-N/ml. were then added, and the volume was made up to 50 ml. with buffer. The final concentrations in this system were thus (mg./ml.): quinic acid, 5.0; MgSO_4 , 0.05; bacterial-N, 0.02. The flasks were prepared in duplicate and placed on the shaker at room temperature. Samples were taken at 30 min. intervals for ferric chloride tests and u.v. absorption spectra. Samples for u.v. spectra consisted of 1.0 ml. samples added to an equal volume of 1.2% (w/v) trichloroacetic acid. The samples were then diluted to 10.0 ml., centrifuged for 15 min., decanted, and diluted with distilled water as

required for determination of their u.v. spectra, illustrated in Fig. 2. Although absorption above $250\text{ m}\mu$ by trichloroacetic acid was negligible at the concentrations used ($D_{257}=0.03$, $D_{293}=0.01$), the zero time reading at $293\text{ m}\mu$ was subtracted in concentration determinations. The blank cell contained distilled water. The quantitative estimation of protocatechuic acid production in the same experiment is given in Fig. 3.

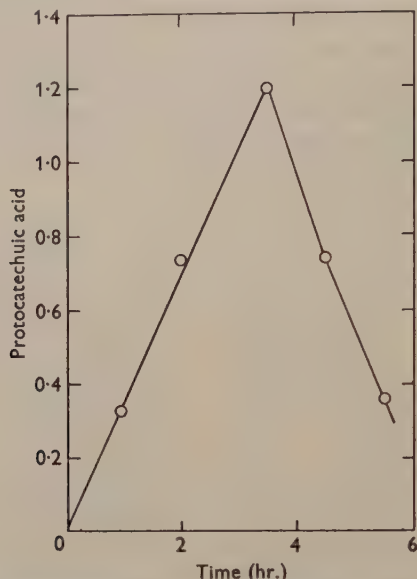


Fig. 3. Production of protocatechuic acid from quinic acid (5.0 mg./ml.) by a washed suspension of organism A-5.

*Identification of protocatechuic acid and production from
p-hydroxybenzoic acid*

In shaken-flask experiments with 0.3% (w/v) *p*-hydroxybenzoic acid as carbon source, no intermediate was apparent by ferric chloride test or u.v. spectrum during incubation for 96 hr. In other experiments 150 ml. volumes of the same medium in 300 ml. Erlenmeyer flasks were inoculated with 1.0 ml. of *p*-hydroxybenzoic acid-grown liquid cultures of the three test organisms, and incubated at room temperature without shaking. Samples drawn from these flasks between 48 and 72 hr. of incubation gave a pale green colour with ferric chloride. The u.v. spectra of samples removed during the same period did not reveal the presence of protocatechuic acid. Protocatechuic acid was not identified in this case by u.v. spectra alone since *p*-hydroxybenzoic acid absorbs much more strongly in the u.v. region than protocatechuic acid; its main absorption maximum is at $255\text{ m}\mu$, and masks the protocatechuic acid maximum at $257\text{ m}\mu$.

Further experiments were carried out with triplicate sets of flasks for each test organism. One flask of each triplicate set was used at 48, 60 and 72 hr., respectively. Metabolism was stopped by heating the cultures to 78° for

10 min. After 72 hr. the three flasks from each test organism set were pooled and centrifuged. The supernatant fluids were acidified and extracted with 3×25 ml. portions of ether. The ether extracts were extracted with 2×20 ml. portions of 10 % (w/v) NaHCO_3 . The aqueous phases were acidified with hydrochloric acid (1 vol. conc. HCl + 1 vol. water) and extracted with 2×25 ml. portions of ether and the ether then removed by warming under a stream of warm nitrogen. Approximately 750 mg. of material was recovered in this manner from a triplicate set of flasks.

Ultraviolet spectra of the recovered material showed an absorption maximum at $293 \text{ m}\mu$; the secondary maximum of protocatechuic acid appears at this wavelength. The main peak in these spectra was at $255 \text{ m}\mu$, characteristic of *p*-hydroxybenzoic acid. By assigning the $293 \text{ m}\mu$ peak to protocatechuic acid, calculation showed the material to contain approximately 4.0 % protocatechuic acid. Infrared spectra of the materials did not reveal any bands other than those characteristic of *p*-hydroxybenzoic acid. Concentration of protocatechuic acid in this material was carried out by a differential solubility procedure. A 500 mg. portion of sample was treated with 3 ml. water and thoroughly mixed. The sample was centrifuged in the cold and the water removed by pipetting. The supernatant fluid was filtered, acidified with 1 drop of '1:1 HCl ' and extracted with two equal volumes of ether. The ether layer was removed and evaporated to dryness. One ml. of water was then added to the residue and the procedure repeated. The final residue was dissolved in 95 % (v/v) ethanol in water and the ultraviolet spectrum determined. Spectra on these samples showed a definite absorption maximum at $293 \text{ m}\mu$, from which a ratio of 35 % protocatechuic acid : 65 % *p*-hydroxybenzoic acid was calculated; the actual values were 4.9 mg. *p*-hydroxybenzoic acid and 2.64 mg. protocatechuic acid (these figures are for culture E-20 fermentation).

Infrared spectra of the enriched samples of material were used to establish the identity of protocatechuic acid as a component of it. Spectra of the pure compounds were determined. Bands characteristic of protocatechuic acid, and distinct from those of *p*-hydroxybenzoic acid, appear at 3.1 , 6.55 , 6.9 , 7.25 , 8.95 , 10.63 , 11.27 and 13.48μ . In the mixed samples distinct bands appeared at 3.1 , 8.95 , 10.63 , 11.27 and 13.48μ ; the 6.55 band appeared as a shoulder. The bands at 6.9 and 7.25μ were distinguishable as extra absorption in these regions. The infrared spectra of the samples were qualitatively identical with that of mixture of 35 mg. of authentic protocatechuic acid with 65 mg. of *p*-hydroxybenzoic acid.

Manometric experiments

A series of experiments was conducted with organisms grown on media containing, respectively, quinic, protocatechuic and *p*-hydroxybenzoic acid, and the same acids as substrates. Non-induced organisms grown with asparagine as sole C source showed a definite lag period with the above compounds as substrates. The results of the experiments with culture A-5 are summarized

in Figs. 4-6; qualitatively equivalent results were obtained with cultures D-19 and E-20.

Growth experiments

Tubes of quinic acid liquid medium were prepared, and tubes containing the same basal medium but with *p*-hydroxybenzoic or *m*-hydroxybenzoic acid as a source of carbon. Tubes of each medium were inoculated with one drop of a 24 hr. quinic acid liquid culture of each organism. All three organisms showed visible growth in the quinic and *p*-hydroxybenzoic acid media and subcultures showed continued growth through five transfers. No growth with *m*-hydroxybenzoic acid as carbon source was observed in any instance.

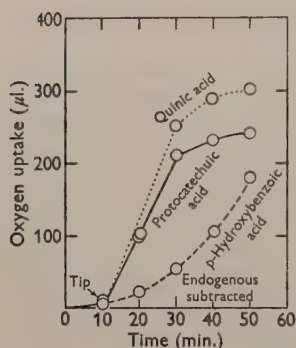


Fig. 4

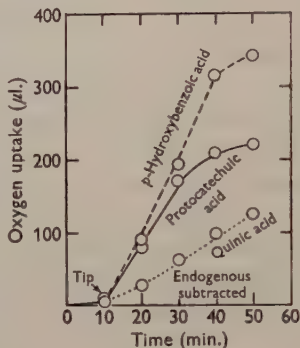


Fig. 5

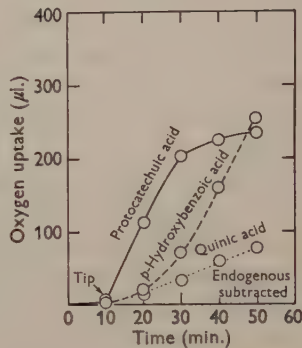


Fig. 6

Fig. 4. Warburg study—resting cells of quinic acid grown culture A-5. Substrate concentration = 3 μ mole.

Fig. 5. Warburg study—resting cells of *p*-hydroxybenzoic acid grown culture A-5. Substrate concentration = 3 μ mole.

Fig. 6. Warburg study—resting cells of protocatechuic acid grown culture A-5. Substrate concentration = 3 μ mole.

DISCUSSION

Culture A-5 used in these studies closely resembles and may be considered a strain of *Pseudomonas aeruginosa*. Culture D-19, although bearing some resemblance to the hydrocarbon-attacking *P. arvilla*, is not identical with that organism; it apparently belongs to the non-proteolytic group of aromatic ring-attacking pseudomonads. Culture E-20 differs from *Achromobacter cycloclastes* in that it did not produce a pale buff pigment on agar slopes. It is doubtful whether species assignment for cultures D-19 and E-20 should be made on the basis of present data.

The production of protocatechuic acid from quinic acid has been confirmed, the conversion being carried out by a wider range of bacteria than hitherto reported. Protocatechuic acid appears as an intermediate in the oxidative pathway, as evidenced by its eventual disappearance from shaken-flask cultures and washed suspensions, and by the fact that quinic acid-induced organisms (Fig. 4) oxidize the compound with no lag period. Protocatechuic

acid production may represent a slow step in the quinic acid oxidation path.

Since an aromatization pathway to *p*-hydroxybenzoic acid, proceeding through 5-dehydroquinic, 5-dehydroshikimic and shikimic acids was known (Davis, 1955, Fig. 7) and an oxidative pathway for *p*-hydroxybenzoic acid proceeding via protocatechuic acid was also known (Evans, 1956; Henderson, 1957), it was felt that *p*-hydroxybenzoic acid might also be an intermediate in quinic acid oxidation. Although *p*-hydroxybenzoic acid was readily attacked by the cultures tested, and it was demonstrated that protocatechuic acid is

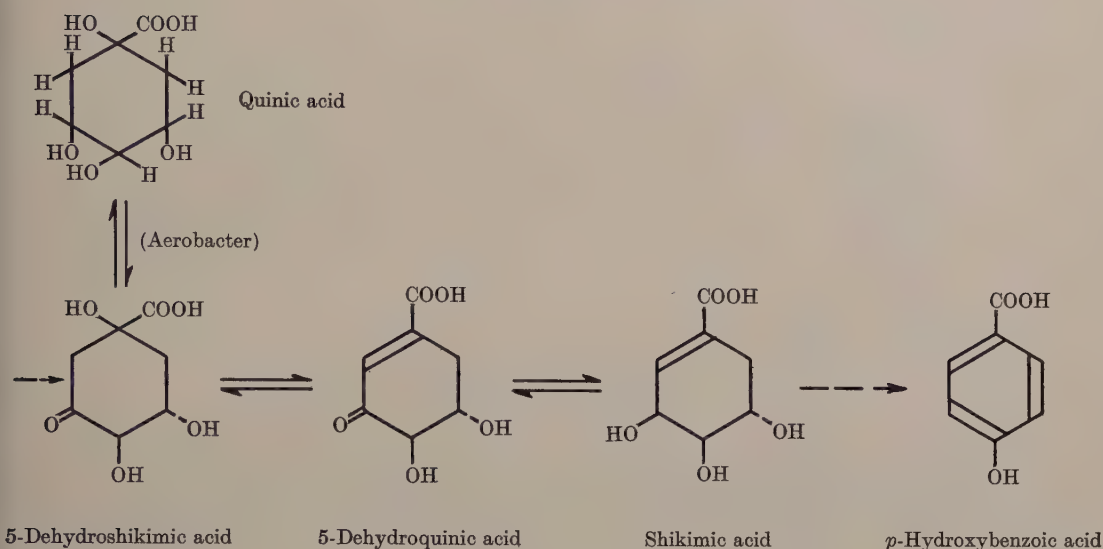


Fig. 7. Aromatization of quinic acid (after Davis, 1955).

indeed produced as an intermediate in *p*-hydroxybenzoic acid oxidation, the sequential induction experiments indicate that the compound is not an intermediate in quinic acid oxidation (Fig. 8); quinic acid-grown organisms showed a lag period before oxidizing *p*-hydroxybenzoic acid, but possessed the enzymes necessary to oxidize protocatechuic acid (Fig. 4). The reaction series is the result of induced enzyme formation since protocatechuic acid-grown organisms do not possess the enzymes necessary to oxidize quinic acid (Fig. 6), and asparagine-grown organisms do not possess the enzymes necessary to oxidize any of the compounds tested.

It is interesting that aromatization of quinic acid occurs in a catabolic pathway, preceding ring splitting and terminal oxidation. Such a pathway would, however, provide a rapid yield of energy from the dehydrations and dehydrogenations necessary to affect such a transformation. Although quinic acid does not lie directly on the known aromatization pathway (Davis, 1955), it is known that an enzyme (quinic dehydrogenase) present in *Aerobacter* converts quinic to 5-dehydroquinic acid (Davis & Weiss, 1955). It is likely

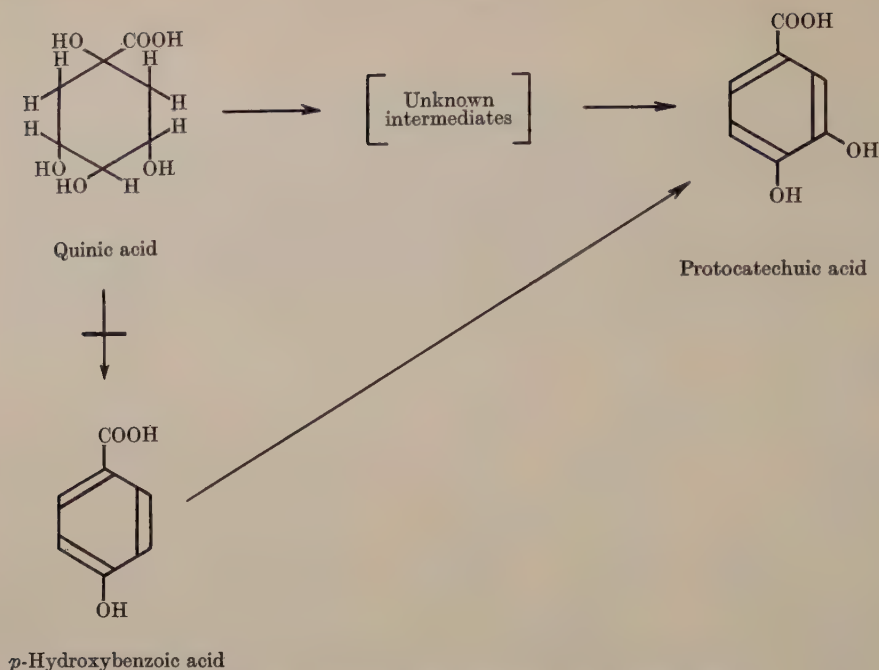


Fig. 8. Protocatechuic acid formation from quinic acid and *p*-hydroxybenzoic acid.

that such an enzyme is present in the organisms under study here, although this has not been demonstrated. Aromatic intermediates which might arise from quinic acid before protocatechuic acid have thus far not been detected.

This paper is No. 4 in the series: Microbiology of Coal.

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'Shortened Latency' as a Result of Multiple Infection by Vesicular Stomatitis Virus in Chick Cell Culture

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SUMMARY: Above a value of one, repeatedly doubling the multiplicity of infection of chick embryo cells by vesicular stomatitis virus progressively shortened the latent period by about 0.6 hr.; this phenomenon is referred to as 'shortened latency'. Varying the multiplicity above unity with dilute-passage stocks did not interfere with rate of infective virus release, number of cells infected, or final yield, i.e. there was no 'von Magnus' effect or other obvious interference phenomena.

The doubling time for virus release was also about 0.6 hr. This suggested that virus may have been growing as a simple intracellular pool equally accessible to all adsorbing virus, and that 1 particle was released when the pool reached a certain size (perhaps 20-200 units) irrespective of inocula. However, other explanations are possible, and of those allowing experimental test, earlier initial adsorption of virus, multiplicity reactivation amongst a partly inactivated population, more rapid elution of attached virus or more rapid release of accumulated internal virus could not account for shortened latency.

Earlier results (Cooper, 1957*a*) suggested that the release of vesicular stomatitis (VS) virus from completely infected chick embryo cell monolayers occurred earlier when inocula of higher titre were used. More specifically, the latent period (defined here as the time between addition of virus and release of one plaque-forming unit or pfu per infected cell) was shorter at higher multiplicities of infection. Final yields and rates of release were not affected.

These findings are confirmed and extended below. For the present purpose, such a phenomenon resulting solely from varying the multiplicity of infection of dilute-passage stocks (other conditions such as temperature and pH being equal) will be referred to as 'shortened latency'. The effect of undiluted-passage stocks will be considered elsewhere (Cooper & Bellett, to be published). Some quantitative aspects of shortened latency are presented, which have suggested a simple hypothesis for the intracellular increase of viral units; this is elaborated in the discussion of this paper. However, there are several alternative explanations, and an attempt is made to evaluate some of them experimentally.

The possible existence of auto-interference and exclusion, so that not all adsorbed virus may participate in viral reproduction, is very relevant to any explanation of shortened latency. This question is considered separately for VS (Cooper, 1958), and the results suggest that homotypic exclusion does not occur with dilute-passage stocks of this virus. Extensive discussion of exclusion is therefore omitted from the present paper.

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METHODS

Viral assays and stocks, one-step growth curve methods and media were as described by Cooper (1957*a*). When infected monolayer cells were removed with trypsin for virus release in suspension or for assay of infective centres, the inoculum was removed, plates washed twice with phosphate buffered saline (PBS, Dulbecco & Vogt, 1954), 2 ml. 0.7 mg. Armour crystalline trypsin/ml. or 2.5 mg. Difco trypsin/ml. added at 37° for 5 min., cells quantitatively resuspended and chilled, washed twice in PBS, and resuspended in 1 ml. of PBS. Large clumps were allowed to settle for 5 min., and the mono-disperse supernatant fluid, containing not less than 50 % of the total cells, was removed for assay.

'*Multiplicity of infection*' relates to the number of pfu adsorbed per cell, and not necessarily to the pfu which actually achieve infection. The multiplicities reached in Figs. 1 and 2 are not dependent upon the plating efficiency of the virus assays (defined as ability to detect infective virus) as the adsorptions for both were carried out in the same manner; an agar cell-suspension method (Cooper, 1955) appeared to detect only 20–40 % more infective virus than did the monolayer method. Therefore 'pfu adsorbed' is regarded as the same as pfu added in the inoculum.

RESULTS

Effect of multiplicity of infection on latent period

Figures 1 and 2 confirm in more detail the earlier suggestion (Cooper, 1957*a*) that higher multiplicities of infection gave shorter latent periods with VS virus growing in chick cells. Thus at higher average multiplicities of infection, on the average each infected cell released its first progeny particle sooner. Rate of release and final yield were not affected; it is therefore noteworthy that no interference phenomena (decrease in release rate or final infective yield) were found. This is characteristic of the dilute-passage stocks used; undiluted-passage stocks show marked interference (Cooper & Bellett, to be published). The experiments shown used the New Jersey serotype, but the same phenomenon was also found with the Indiana serotype.

The experiments of Figs. 1 and 2 were performed with chick cell monolayers of the same batch infected simultaneously with different dilutions of the same high-titre virus stock, thus excluding possible day-to-day variations in cells or virus preparations as the explanation of apparent shortened latency. Shortened latency is not due to re-adsorption effects (possible in the monolayer experiment of Fig. 1) as identical results were obtained in dilute cell-suspensions (Fig. 2) where re-adsorption of released virus was negligible. This similarity also suggests that, in general, re-adsorption is not a significant factor in monolayer release curves, which is to be expected from calculations involving adsorption rates (50 % of virus is adsorbed in 3 hr. from 5 ml., whereas release is doubled every 30 min.). Figure 3 shows that there was a roughly logarithmic relation between multiplicity and latent period; a possible

significance of this is discussed later. The experiment of Fig. 2 (at 37°) was repeated with the exponential release period at 30° (the latent period having been at 37° as before), when the same shortened latency occurred even though the release doubling time was lengthened to approximately 1 hr.

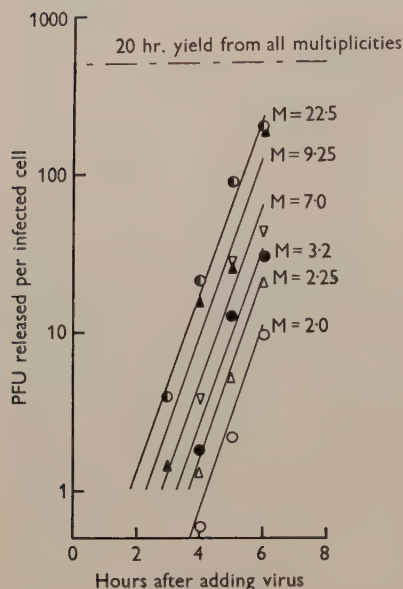


Fig. 1

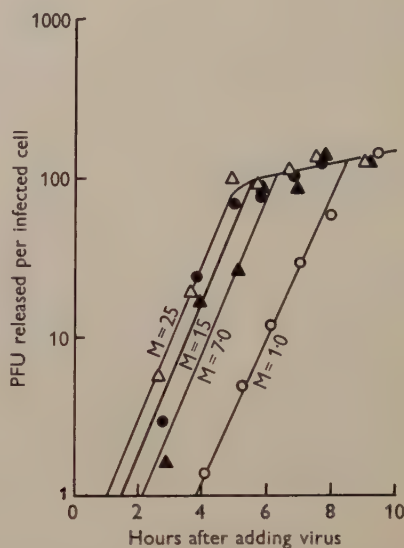


Fig. 2

Fig. 1. Effect of multiplicity of infection on one-step virus release curves from intact monolayers of the same batch (average pfu/infected cell). Virus was adsorbed for 45 min. from 0.5 ml. by monolayers of 2×10^7 cells, which were then washed 3 times with 5 ml. of medium. Free virus then assayed less than 1 pfu/10 infected cells. Release was allowed to proceed into 10 ml. medium from intact monolayers. Multiplicities of adsorption were 2.0 (○), 2.25 (△), 3.2 (●), 7.0 (▽), 9.25 (▲) and 22.5 (◐) pfu/cell. The multiplicities of adsorption 3.2–22 were sufficient to infect all cells in the first cycle; correction in the two lower curves for cells not infected ($pr=0.176$ and 0.135) gave average multiplicities of infection of 2.0 and 2.25, and the curves (but not the points) have been corrected for these infective centre errors.

Fig. 2. Effect of multiplicity of infection on one-step virus release curves from cells in suspension. After infection of the monolayers (all of the same batch) to known multiplicity, cells were rapidly removed with trypsin, washed twice with medium and resuspended in medium at 37° to 10^4 cells/ml., while still early in the latent period. At intervals, samples of medium+cells were frozen unseparated for virus assay. Multiplicities of adsorption were 1.0 (○), 7.0 (▲), 15 (●) and 25 (△) pfu/cell. The culture with the lowest multiplicity of absorption (1.0) received only 0.15 pfu/cell in the monolayer. Free virus was initially about 10% of the concentration of infected cells. Plating efficiencies (infective centres/total cells) were about 60%, except for multiplicity=1, which was 10%.

Possible explanations for 'shortened latency' shown to be unlikely

Virus elution. Virus may elute from the higher multiplicities of adsorption, giving apparently earlier release; in this case the earlier liberation of virus would be the summation of elution and virus release from an effectively low- or single-multiplicity infection. It is necessary to postulate for this either that

the probability of elution of each adsorbed particle is much greater in multiple than in single infections, or the unlikely case that higher multiplicities depress the final yield by a factor exactly equal to the proportion of virus eluting from lower multiplicities, since elution from low multiplicities would reduce the number of infective centres.

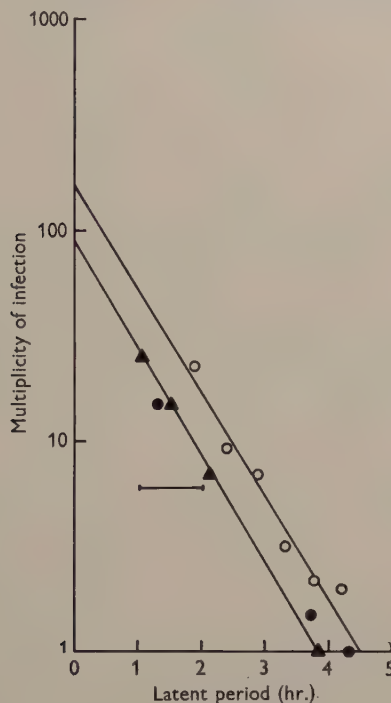


Fig. 3. The relationship between multiplicity of infection and latent period derived from Fig. 1 (○) and Fig. 2 (▲), which each used plates of one batch. Three points (●) are from independent experiments with different batches of plates, and the bar at multiplicity of six represents the extreme range of latent periods observed in Fig. 1 of Cooper (1957*a*).

That elution is not occurring is shown by: (*a*) the exponential nature of the release curves yielding virus in significant excess over that added, and (*b*) when virus growth (in a suspension containing in 1 ml. 10^4 cells previously infected in monolayers to an average multiplicity of 20) was prevented 40 min. after infection by freeze-thaw disrupting the cells or by the addition of 10 mM-NaCN, the 10 hr. virus yield/cell and therefore presumably the elutable virus was less than 0.1 % of the yield/untreated cell. In a number of experiments, the pfu liberated after 1 freeze-thaw of cells infected with high multiplicities was equivalent to 1–10 % of the number of infected cells, or less than 1 % of the virus added (Cooper, 1958); this is of the same order as the intact cells probably present (Cooper, 1957*b*).

Earlier adsorption of a single particle. As the rate of VS virus adsorption is probably independent of virus concentration, on average the time at which each cell receives its final quota of virus should be constant and independent

of multiplicity. However, a higher virus titre in the inoculum would mean that each cell would be likely to receive its first particle in a shorter time. It can be shown that little more virus is adsorbed by monolayers after 30 min. under the conditions used and 50 % is adsorbed in 10–15 min., independently of the concentration; therefore for the longest latent period, i.e. for single infection, half the cells have received their virus by 15 min. and nearly all by 30 min. The inoculum was usually removed and the plates washed at this stage. Thus the higher titres of virus added for the higher multiplicities could not achieve single infection more than 30 min. before the low titres. The latent period could not therefore be shortened in this way by more than 30 min., and there should be little difference in latent periods above multiplicity 2–3; reference to Figs. 1 and 2, where the maximum shortening was 3 hr., and where continuing increase in the multiplicity above 3 still further shortened the latent period, shows that shortened latency is not due to earlier adsorption at higher multiplicities.

Multiplicity reactivation among a partly inactivated virus population. It is very likely from the thermal instability of VS virus (Cooper & Bellett, to be published) that freshly harvested virus preparations contained an excess of virus which was non-infective for this system (i.e. not leading to infective progeny and therefore a plaque); many preparations certainly contained an appreciable proportion, often an excess, of particles 'storage'-inactivated during sojourn at -20° . It can be imagined that these may yield infective progeny under conditions of multiple infection and thereby affect the latent period in certain circumstances. However, direct examination for multiplicity reactivation in the sense of larger recovery of infective centres at high multiplicities of infection compared with low, revealed none in a population containing a tenfold excess of 'storage'-inactivated and an unknown excess of thermally inactivated particles (Table 1). Alternatively, the penetration of the cell by several particles, active or inactive, may provide material, perhaps structural protein or enzymes, which can overcome some bottleneck in virus production and lead to earlier maturation. In this case pretreatment of the cells with inactive particles might affect the latent period. Reference to Fig. 1 of Cooper (1958), where the cells were pretreated with a large excess of 'storage' and uv-inactivated particles, shows no effect on latent period by this means. Similar experiments (Cooper & Bellett, to be published) with an excess of the interfering component present in undiluted-passage stocks also showed no effect on the latent period in those cells able to release virus, and numerous one-step growth curves at single multiplicity (using various stocks and an input multiplicity of 10^{-3} to 0.3) always gave a latent period of 4.0–4.5 hr., showing that the varying multiplicities of thermally inactivated virus thereby achieved can have no marked effect on latent period.

Earlier release of an accumulated pool of mature virus

Experimental manipulation of cells infected with virus have in some hands stimulated virus release; there is evidence that mature poliovirus may accumulate in or on cells (Howes & Melnick, 1957) and be released in a burst (Lwoff,

Table 1. *The lack of effect of dilution of a dilute-passaage seed containing excess of 'storage'- and thermally-inactivated virus on the recovery of cells yielding infective progeny (lack of multiplicity reactivation)*

Dilutions of a VS virus stock originally assaying 5×10^8 pfu/ml., and reduced to 6×10^7 pfu/ml. on storage at -20° , were added in 0.5 ml. amounts to each of 7 monolayers containing 2×10^7 cells; after 45 min. the inoculum was removed, plates washed twice with 5 ml. Earle's saline, cells removed with trypsin, chilled, washed once with PBS, re-suspended in 1 ml. and supernatant removed after 5 min. for assay of infective centres on monolayers.

Dilution	Seed		Assay			Infective centres per monolayer		Plating efficiency (%)	
	Multiplicity of infective VS (pfu)	Minimum multiplicity of inactivated VS	Dilution	pfu in		Observed	Expected from pfu added in seed	Observed infective centres	Expected infective centres
				0.1 ml.	0.2 ml.				
Neat	1.5	11	5×10^{-5}	17, 10	—	2.7×10^6	1.08×10^7	25.0	
1/3	0.5	3.6	5×10^{-5}	10, 9	—	1.9×10^6	5.6×10^6	34.0	
1/10	0.15	1.1	10^{-4}	5, 6	—	5.5×10^5	2.5×10^6	22.0	
1/30	0.05	0.36	3×10^{-4}	11, 10	13, 18	2.9×10^5	7.5×10^5	38.7	
1/100	0.015	0.11	10^{-3}	8, 16	20, 22	1.1×10^5	2.5×10^5	44.0	
1/300	0.005	0.036	3×10^{-3}	8, 13	13, 14	2.7×10^4	7.5×10^4	36.0	
1/1000	0.0015	0.011	10^{-2}	6, 5	15, 16	7.0×10^3	2.5×10^4	28.0	

Dulbecco, Vogt & Lwoff, 1955). High multiplicities of infection with VS virus may possibly have so damaged the cell surface that an accumulated pool of infective virus was released sooner than with low multiplicities. However, this would mean that the non-released infective virus associated with singly infected cells must be at least as numerous as the virus released by the higher multiplicity. Thus non-released virus during single infection would have to exceed the released virus by a factor of 10 or more. In fact, precisely the opposite was found by Franklin (1958) for VS virus growing in chick and monkey-kidney cells, i.e. the released virus exceeded the non-released by a factor of 10, as was found for western equine encephalomyelitis virus by Rubin, Hotchin & Baluda (1955). This implies that any virus particle must be released very rapidly once it is mature (becomes infective).

Further evidence against this idea is that in the experiment of Fig. 2 at least 90 % of cells were ruptured before assay by freezing and thawing, so that any non-released pool would be artificially released and included in the total count; it can be seen that the total virus produced by the cells (released plus non-released) still shows shortened latency.

DISCUSSION

Some data of Doermann (1952) suggested that intracellular coliphage T4 appeared slightly earlier with higher multiplicities, although the absence of marked shortened latency in bacteriophage may be explained by the presence of mutual exclusion. Dulbecco & Vogt (1954) found that the latent period of western equine encephalomyelitis virus was shorter at a higher multiplicity. Liu & Henle (1951) found a similar phenomenon with influenza virus (strain LEE), although this might be accounted for by an enzymic release of attached virus in the same way that multiplicity reactivation was simulated (Henle & Liu, 1951), but later shown probably not to occur in this sense (Cairns, 1955). Kaplan (1957) mentioned an earlier release of herpes simplex virus at higher multiplicities, but as, due to technical difficulties, he appeared in some doubt as to the precise number of infected cells present in these experiments one cannot say whether the latent period, as defined in the present paper, was shortened. Darnell (1958) presented data which suggest a longer latent period in single than in high multiplicity for intracellular poliovirus. However, the reverse applied among the multiple infections (i.e. the latent periods were longer for higher multiplicities) and separate infective centre assays (particularly needed for the single multiplicities) were not given, so that shortened latency was not demonstrated. Dr R. M. Franklin (personal communication) found that L cells, infected with fowl-plague virus and subsequently 'stained' with fluorescent antibody, fluoresced earlier the higher the multiplicity.

The results presented above confirm the existence of the phenomenon of shortened latency in VS virus-infected chick cells. Certain possible explanations of the phenomenon seem to be excluded, namely, elution of virus at the higher multiplicity, earlier infection of the culture with seeds of higher titres, multiplicity reactivation of inactivated virus or pooling of certain

components to produce a complete particle sooner, and an earlier release of an accumulated pool of mature virus at the higher multiplicities. These will not be considered further.

It is demonstrated elsewhere (Cooper, 1958) that homotypic exclusion probably does not occur with dilute-passage VS stocks, although undiluted-passage VS stocks can contain a possibly 'incomplete' form which can exclude homotypic infective VS (Cooper & Bellett, to be published). However, all stocks used had only a limited number of passages (less than three, all with dilute inocula in tissue culture) from a picked single plaque, had a high titre ($1-2 \times 10^9$ pfu/ml.) and showed no depression of yield at higher multiplicities of infection, and so it is believed that they contain negligible amounts of the excluding component. There seems therefore no direct evidence for believing that all adsorbing particles are not participating, at least to some extent, in the growth cycle; until information on homotypic exclusion can be obtained, for example, from genetically labelled strains little further can be said on this subject.

It is proposed to consider briefly some explanations of shortened latency which assume non-exclusion and which did not allow direct experimental test. The earlier intracellular appearance of the first infective virus particle in multiple infection suggests that entry of several particles: (a) is equivalent to one particle reproducing for longer, (b) enables one particle to start reproducing sooner, or (c) permits one particle to reproduce faster. Possibilities (b) and (c) need not imply mutual exclusion between particles entering a cell, and all three possibilities are not mutually exclusive; (a) and (b) may well occur together.

If (a) is true, for example by multiple contributions to a vegetative pool (suggested by recombination among certain other animal viruses), then the following hypothesis is possible.

Let M = number of contributions to the pool (multiplicity of infection) and assume that increase of viral multiplying units is exponential.

Then r = the number of multiplying units present at the end of the latent period = $M \cdot 2^k$, where $k = (L - d)/g$, and L = length of latent period, d = 'dead-time', during which the virus particle adsorbs, penetrates and organizes into a replicating state, g = doubling time of pool.

Therefore $\log M = -L/g \log 2 + d/g \log 2 + \log r$.

A plot of $\log_{10} M$ against latent period (Fig. 3) gave a straight line of negative slope, indicating that if d is constant during either of the two series of growth curves of Fig. 3 then r is constant for the multiplicities studied. The value of r cannot be determined as d is unknown but the extrapolation to zero latent period indicates that r is less than 200. The ending of some latent periods by 1-1.5 hr. means that multiplication has at least started in these cells by this time, and if d is constant for all multiplications then d is less than 1.5 hr. and r is greater than 20. Calculations of g from both slopes in Fig. 3 gave values of about 0.6 hr., similar values to those calculated for the release doubling time from the virus release curves (0.54 and 0.60 hr. respectively; most other experiments gave 0.6 hr.).

In other words, doubling the multiplicity should put the pool one generation

ahead. Therefore in the simple case where final yield and rate of multiplication are unaffected, where the content of multiplying units is constant (20–200) when an average of one particle is released per cell, where vegetative growth and release have the same exponential rate, and where the pool is equally accessible to all adsorbing particles, then doubling the input should decrease the latent period by one release-doubling time. These assumptions are all implicit in this hypothesis, which then suggest a rather low probability of maturation of vegetative units compared with the high probability of release of mature virus found by Franklin (1958).

Possibility (b) may be caused by co-operation between particles in penetration or in organizing a replicating site, or in merely giving a greater probability of success in penetrating or in intracellular collision with a suitable site. Cairns (1957) suggested that in influenza virus infections of the allantoic membrane there is a probability of delay after adsorption in starting an infection at single multiplicity, so that each of a series of such infections will start multiplying at very different times. It is likely that a similar probability of delay occurs among particles multiply infecting a cell, so that the higher the multiplicity the sooner virus growth might be started by one of the adsorbing particles. Possibility (c) is allowed because exponential release may be the result of a series of 'bursts' randomly arranged among a large population of cells, and the actual duplication rate need bear no relation to release. It was mentioned above that an accumulation of mature virus was not found, making the 'burst' hypothesis for release alone unlikely, but several ways can be envisaged in which wholesale maturation and release could occur very rapidly in individual cells.

These questions are fundamental to our concept of viral growth. Means to investigate some of the alternatives discussed above could be devised, but some more direct method is very desirable.

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Homotypic Non-exclusion by Vesicular Stomatitis Virus in Chick Cell Culture

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SUMMARY: Placing live or inactivated vesicular stomatitis virus of one serotype on chick cells in tissue culture prevented most of the cells from releasing infective virus of the other serotype when super-infected with it (heterotypic exclusion). Inactivated virus did not prevent super-infection with the same serotype and had no effect on the latent period or rate of virus release (homotypic non-exclusion and non-interference). The 'Indiana' serotype was more effective as heterotypic excluding agent than was the 'New Jersey' serotype, and exclusion was noticeable when only 12 min. elapsed between interfering and challenge virus. Each cell liberated virus of only one serotype when infected with live virus of both serotypes, but the serotype released was often (20-40 %) not that of the particle first adsorbed. Heterotypic exclusion in fact behaved as if it were reversible and dependent on the multiplicities of infection, at least within the latent period. Many inactivated particles per cell were adsorbed before heterotypic exclusion was achieved.

The phenomenon of 'shortened latency' in vesicular stomatitis (VS) virus infections of chick embryo cells (Cooper, 1958), where a higher multiplicity of infection caused the first infective progeny to appear sooner, raised the question of whether one particle of this virus was able to exclude an identical particle from infecting the same cell. Clearly any interpretation of shortened latency is affected by exclusion, since then all adsorbing virus would not be contributing equally to the progeny.

This paper demonstrates the presence of heterotypic exclusion by live or inactivated preparations of VS virus (Indiana or New Jersey serotypes) under conditions where the same inactivated preparations do not exclude the live homotypic virus. The conclusion is drawn that early exclusion between live homotypic particles is therefore also unlikely, although not disproven.

Some interpretations of shortened latency are discussed elsewhere (Cooper, 1958); because of the particular relevance to shortened latency the present paper examines interference mostly in the early latent period.

METHODS

Virus stocks were high titre dilute-passage stocks containing minimal amounts of the non-infective interfering component (Cooper & Bellett, 1958), and were prepared by growth on chick embryo cell monolayers as described by Cooper (1957). Virus titres were obtained by plaque assays on chick embryo mono-

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layers as described in the latter paper. The guinea-pig hyper-immune antisera used in separating the serotypes (kindly given by the Research Institute, Pirbright) had no effect on plaque counts of their respective serotypes when incorporated to a concentration of 1/6000 (v/v) in the agar overlay, but completely prevented plaque formation at 1/600; a concentration of 1/120 was routinely used, and its effectiveness usually checked for each experiment. Plaque counts with heterotypic antisera of either type were completely unaffected at 1/60 (v/v).

Ultraviolet inactivation was accomplished by irradiating 1 or 2 ml. of tissue culture fluid for 1–5 min. in a 10 cm. Petri dish at 18° on an automatic rocking device which tilted the dish once every 2 sec. Under these circumstances one lethal hit occurred about once every 40 sec. The dose for distilled water was about 5 ergs mm.⁻² sec.⁻¹.

Technique for establishing conditions of exclusion

All experiments were made by adding a primary inoculum to 20 hr. chick embryo monolayers (usually the interfering agent) followed after a period of adsorption by removal of the inoculum, addition of the secondary inoculum (usually the challenge virus) and its removal after a second period of adsorption. These are described where appropriate. Usually cells were washed and removed by trypsinization for assay of infective centres as described below; exclusion is therefore defined and used in the bacteriophage rather than the sperm-ovum sense, i.e. a prevention of a potentially adsorbing and infecting virus from liberating infective progeny characteristic of its own serotype, and therefore from forming a plaque neutralized by its own antiserum.

All operations up to cell chilling were made in an incubator hood, which is a Perspex box with sloping front and sleeved arm-holes designed for manipulating Petri dish monolayers in an atmosphere of controlled temperature, CO₂ content and humidity. Thus temperature, pH and tonicity variations in the monolayers were eliminated during the early latent period, which was both the time under investigation and the time potentially affected by the manipulations. The similarity of one-step growth curves produced in the incubator hood with those produced without it showed that such variations are not important for growth curves provided that they are soon corrected (usually within 15 min.); their elimination was important for the present purpose as otherwise they might be incriminated as potential causes for the lack of homotypic exclusion described below.

Harvesting cells for assay. After the final adsorption of seed, fluid was aspirated and drained as completely as possible, plates were washed twice with PBS (phosphate buffered saline, Dulbecco & Vogt, 1954), and cells removed by 5 min. treatment with 5 ml. trypsin (2.5 mg./ml. in PBS) at 37°. The suspension was thoroughly chilled in ice, centrifuged, washed once in PBS and suspended in 1 ml. cold 50 % (v/v) PBS in 'conditioned' medium (centrifuged medium from formed 20 hr. monolayers). After 5 min. standing to settle large clumps, the supernatant 0.75 ml. was removed for assay of infective centres and total cells (haemocytometer count). The suspension was

then almost entirely monodisperse. Cells were plated while still in the early latent period; after all plating of cells was complete, free virus was estimated as the infective centres remaining after 3 min. centrifuging at 1000 rev./min. Some estimation of the higher limit of 'associated' virus, i.e. externally attached plus internally contained mature virus, was obtained as the number of infective centres remaining after one cycle of freezing at -70° and thawing; the relevance of this control is that the externally attached virus may elute and give spuriously high infective centre counts. Such elutable and free virus was always low compared with the total infected cell count. It is felt that the bulk of the 'associated' virus in the present work is accounted for by the undisrupted cells still present (up to 10 % of the total).

RESULTS

Heterotypic but not homotypic exclusion by inactivated VS virus

Table 1 shows that when monolayers of chick cells from the same batch were allowed to adsorb a high multiplicity of 'storage'—(-20°) plus ultraviolet-inactivated (UVI) virus particles of either New Jersey (NJ) or Indiana (IND) serotype, 100 % of the cells thus infected could produce progeny from a single homologous active particle added subsequently, but only 20 % (IND VS as primary inoculum) or 40 % (NJ VS as primary inoculum) from a single subsequent heterologous particle. This lower efficiency of NJ VS as an interfering agent was evident in all of three experiments where it might be found, despite a frequently higher multiplicity of infection by the NJ VS.

In these experiments the interfering agent was given the greatest opportunity of establishing itself, consistent with limitation to the early part of the latent period, namely, adsorption for 1 hr., frequent resreading of the inoculum, and high multiplicity of infection. It can be seen that, despite this maximum opportunity, the heterotypic exclusion is not efficient in the sense that by no means all the cells having the interfering agent are prevented from producing progeny. The primary inoculation was carried out by a method which had been shown with live virus to infect nearly all of the cells (Cooper, 1955, 1957), so that all cells are likely to receive inactivated virus. Nevertheless, although heterotypic exclusion is not complete, its presence in 60–80 % of the cells rules out the possibility that the non-appearance of homologous exclusion in the same experiment was due to some failure of the majority of cells to be infected by the primary inoculum, for example, that the inactivated virus did not adsorb.

Since these preparations are certain to contain much heat-inactivated virus (the half-life of VS at 37° is 1–3 hr. in the usual growth media, Cooper & Bellett, to be published), the lack of homotypic exclusion applies to heat-inactivated as well as to UVI and storage-inactivated virus.

The heterotypic exclusion by inactivated NJ or IND strains was reciprocal, but whether 'mutual exclusion' occurs in the sense that only one but not both serotypes can grow together in the same cell will be discussed below. As was found repeatedly in other experiments, no multiplicity reactivation was

Table 1. *Effect of pretreatment with homotypic inactivated VS virus on infected cell count*

As with all tables in this paper, the data relate to one experiment with one batch of plates and virus seeds. The seeds used here initially contained 10^9 pfu/ml. (NJ) and 2.0×10^8 pfu/ml. (IND) and had inactivated to 2.8×10^8 pfu/ml. and 6.1×10^8 pfu/ml. respectively at -20° ; portions of these stocks were then ultra-violet irradiated to 10^{-4} (NJ stock, 9 lethal hits) and 10^{-5} (IND stock, 12 lethal hits) survivors. Primary inocula (0.5 ml.) were respread 3 times during 1 hr. at 37° , after removal of this but without washing,

secondary inocula (0.5 ml.) were added for a further 30 min. when the cells were washed and resuspended to 1 ml. for assay. M = multiplicity of infection with live, or 'stor' = storage and 'UVI' = ultra-violet inactivated virus (the fraction indicates ratio of added infective virus particles to total cells in the monolayer). Plaque assays at 10^{-3} of monolayer 6 with IND anti-serum in the agar overlay gave 2 plaques, and of monolayer 3 with NJ anti-serum gave no plaques.

Mono-layer no.	Primary inocula (inactivated)		Secondary inocula (live)		Total cells re-covered $\times 10^7$	Infective centres per monolayer		
						Cells intact (pfu)		Not sedimented by 3 min. at 1000 rev/min. (pfu at 10^{-3})
	M	Type	M	Type		At 10^{-3}	After freeze-thaw (pfu at 10^{-3})	
1	0	—	1 (1/20)	NJ	2.0	$\left. \begin{matrix} 230 \\ 221 \end{matrix} \right\}$	$\left. \begin{matrix} 4.5 \\ 24 \end{matrix} \right\}$	2
2	20 'stor' + 7 UVI	NJ	1 (1/20)	NJ	1.5	$\left. \begin{matrix} 260 \\ 240 \end{matrix} \right\}$	$\left. \begin{matrix} 5.0 \\ 47 \end{matrix} \right\}$	0
3	45 'stor' + 15 UVI	IND	1 (1/20)	NJ	2.0	$\left. \begin{matrix} 30 \\ 68 \end{matrix} \right\}$	$\left. \begin{matrix} 1.0 \\ 18 \end{matrix} \right\}$	0
4	0	—	1 (1/30)	IND	1.4	$\left. \begin{matrix} 140 \\ 143 \end{matrix} \right\}$	$\left. \begin{matrix} 2.8 \\ 39 \end{matrix} \right\}$	4
5	45 'stor' + 15 UVI	IND	1 (1/30)	IND	1.2	$\left. \begin{matrix} 146 \\ 157 \end{matrix} \right\}$	$\left. \begin{matrix} 3.0 \\ 17 \end{matrix} \right\}$	10
6	20 'stor' + 7 UVI	NJ	1 (1/30)	IND	1.5	$\left. \begin{matrix} 66 \\ 66 \end{matrix} \right\}$	$\left. \begin{matrix} 1.3 \\ 23 \end{matrix} \right\}$	8
7	45 'stor' + 15 UVI	IND	0	—	1.3	$\left. \begin{matrix} 16 \\ 124 \end{matrix} \right\}$ at 10^{-1}	$\left. \begin{matrix} 3.2 \\ 25 \end{matrix} \right\} \times 10^3$	—
8	20 'stor' + 7 UVI	NJ	0	—	1.2			—

evident in Table 1 among UVI or storage-inactivated particles, as the plating efficiency of the cells receiving inactivated virus (monolayers 7 and 8) in terms of the ratio of cells infected (pfu added): infective centres recovered was the same as the controls (monolayers 1 and 4). No reactivation of the inactivated serotype by the active was detected either (monolayers 3 and 6, columns 9 and 10), in contrast to the findings of Gotlieb & Hirst (1956) with influenza; this may be due to the lack of relationship between the two serotypes found serologically (Brooksby, 1949).

Table 2 shows that homotypic UVI virus still had no excluding action when added up to 30 min. after the active virus.

Heterotypic exclusion by live virus

It does not seem possible to show exclusion by live virus directly for any preparation where the inactivated particles exclude and where a content of less than 50 % inactive particles cannot be guaranteed. It is possible that all the reciprocal heterotypic exclusion found in the present work by preparations containing live virus is due to the inactive particles also contained. The most that can be said at present is that wholly inactive preparations behave similarly to preparations containing not more than 50 % active particles, the rest being heat- or storage-inactivated (Table 3). As in Table 1, exclusion is reciprocal between NJ and IND, but IND is the more efficient excluding agent. Free and associated infective virus was negligible.

Minimum time to establish exclusion

Table 3 shows that with NJ VS as the primary inoculum and IND as the secondary, addition of the secondary inoculum only 12 min. after the primary still caused a marked decrease in the number of cells releasing IND VS. The significance of this is discussed below.

Likelihood that heterotypic exclusion is reversible

Heterotypic exclusion is not complete: 20–40 % of the cells infected by the primary serotype yield virus of the secondary serotype. This indicates either that some cells can support growth of both serotypes at once, or that the process of infection is reversible in many cells at the stage at which they have been challenged.

Table 4 shows the results of infecting cells with both NJ and IND serotypes in such a way that the efficiencies of exclusion were about equal, i.e. the less efficient NJ serotype was present in threefold excess of multiplicity. Significant elution of cell-associated virus from infected cells has never been found in these experiments. The only interpretation of the experiment of Table 4 is, therefore, that the secondary serotype overcame the primary in almost precisely half the cells, while mixed yielders were negligible. Mutual exclusion was demonstrated: a cell was able to liberate VS virus of only one serotype, but the primary infection was reversed in half of the population by the secondary serotype. This was so whether the primary serotype was IND or NJ.

Table 2. *Lack of effect on infected cell count of superinfecting with homotypic UVI VS virus (IND serotype)*

The virus stock contained 1.8×10^9 pfu/ml.; the 'active' seed was stock inocula (0.5 ml.) were removed after varying times, plates drained without diluted $1/3$ (giving multiplicity of infection, M , of 24 when added in 0.5 ml. washing and the secondary inocula (0.5 ml.) were added for a further amounts to monolayers containing 1.25×10^7 cells), and the UVI seed was 30–40 min. The cells from each monolayer were then washed and re-stock irradiated to 3×10^5 pfu/ml. (9 lethal hits, giving $M=70$). The primary suspended to 1 ml. for assay.

Monolayer no.	Primary inocula				Secondary inocula			Infective centres per monolayer			
	M	Infectivity	Min. adsorption	M	Infectivity	Min. adsorption	M	Dilution plated	Total pfu		Not sedimented by 3 min. at 1000 rev/min. pfu at 10^{-4}
									pfu	per ml. $\times 10^6$	
1	24	Active	5	70	UVI	40		10^{-5}	9	1.6	3
2	24	Active	14	70	UVI	31		10^{-4}	81		
3	24	Active	30	70	UVI	35		10^{-5}	8	1.8	7
4	24	Active	34	0	—	39		10^{-4}	90		
5	70	UVI	34	0	—	39		2×10^{-5}	30	3.0	7
								2×10^{-5}	24, 19	2.2	16
								10^{-3}	3	0.006	1

Table 3. *Reciprocal heterotypic exclusion between live preparations of IND and NJ serotype VS virus, and its apparently rapid onset*

The primary inocula (0.5 ml.) were allowed to adsorb for the indicated times, when they were removed and the secondary inocula (0.5 ml.) added for 30 min. The cells from each monolayer were then washed and resuspended in 1 ml. for assay. The NJ stock originally contained 1.2×10^8 pfu/ml. and had inactivated to 3.2×10^8 pfu/ml. at -20° ; it was used undiluted or diluted 1/50. The IND stock originally contained 2.2×10^8 pfu/ml. and had inactivated to 7×10^8 pfu/ml. at -20° ; it was used diluted 1/2 or 1/100. 'NJ serotype' and 'IND serotype' represent plaque assays with respectively IND and NJ antisera in the agar overlay. M = multiplicity of infection with live or 'stor' = storage-inactivated (-20°) virus.

Mono-layer no.	Primary inocula			Secondary inocula (M=1($\frac{1}{4}$)). Type	Total cells recovered $\times 10^6$	Cells intact				Infective centres per monolayer		
	M	Type	Min. adsorption			NJ serotype pfu		IND serotype pfu		After freeze-thaw. Total pfu at 10^{-3}	Not sedimented† by 3 min. at 1000 rev./min. Total pfu at 10^{-3}	
						Total pfu at 10^{-4}	at 10^{-3}	per ml. $\times 10^4$	at 10^{-3}			per ml. $\times 10^4$
1	0	—	50	IND	8.0	45 (10^{-3})	0	—	—	9.0	0	0
2	13+ 40 'stor'	NJ	12	IND	8.0	35	—	70	20	4.0	0	0
3	13+ 40 'stor'	NJ	17	IND	8.5	39	—	78	14	2.6	3	1
4	13+ 40 'stor'	NJ	25	IND	8.0	72	—	144	12	3.4	0	1
5	13+ 40 'stor'	NJ	50	IND	8.0	90*	—	180	22	2.9	3	0
6	0	—	40	NJ	9.5	72 } 10^{-3} 77 }	—	14.9	14 15	—	0	0
7	15+ 30 'stor'	IND	40	NJ	9.0	35*	15 3	1.8	—	70	20	25

* The intact cell preparation from monolayers 5 and 7 gave no plaques when plated at 10^{-3} with both antisera.

† Preparations from monolayers 2, 3, 4 and 5 after freeze-thaw or sedimentation and plated at 10^{-3} with NJ antiserum, and from monolayer 7 plated with IND antiserum, gave no plaques.

Table 4. *Distribution of serotype among infective centres produced by mixedly infecting monolayers with live IND and NJ serotypes at multiplicities giving approximately equal efficiencies of exclusion*

The IND stock initially contained 1.8×10^8 pfu/ml., had inactivated to 3.6×10^8 pfu/ml. at -20° and was used diluted 1/3. The NJ stock initially contained 1.2×10^9 pfu/ml., had inactivated to 6.8×10^8 pfu/ml. at -20° and was diluted 1/2. Primary inocula (0.5 ml.) were removed after 30 min., when the secondary inocula (0.5 ml.) were added and left for 30 min. The cells

from each monolayer were then washed and resuspended to 1 ml. for assay. 'NJ serotype' and 'IND serotype' represent plaque assays with respectively IND and NJ antisera in the agar overlay. M = multiplicity of infection with live or 'stor' = storage-inactivated virus.

Mono-layer no.	Primary inocula		Secondary inocula		Total cells recovered $\times 10^6$	Infective centres per monolayer					
	M	Type	M	Type		Total pfu at 10^{-4}	NJ serotype pfu		IND serotype pfu		Not sedimented by 3 min. at 1000 rev./min. pfu at 10^{-3}
							per ml. $\times 10^6$	at 10^{-4}	per ml. at 10^6	at 10^{-4}	
1	10 + 40 'stor'	IND	0	—	12.0	202	3.7	—	—	3.7	43
2	30 + 25 'stor'	NJ	0	—	12.2	249 205	4.5	—	4.5	—	33
3	10 + 40 'stor'	IND	30 + 25 'stor'	NJ	12.8	300 241	5.4	101 115	2.2 145	2.7 124	63
4	30 + 25 'stor'	NJ	10 + 40 'stor'	IND	12.8	200 220	4.4	129 116	2.4 124	2.3 111	71

Table 5 shows that the efficiency of reversal (proportion of secondary to primary serotypes in the total yield of infective centres) was considerably increased when the multiplicity of infection of the secondary serotype was increased. Free and elutable virus was negligible.

Minimum number of particles required for heterotypic exclusion

As the total number of particles (infective plus non-infective) in the VS preparations is not known, and as inactive particles can be shown to exclude heterotypically, any estimate of number of particles required for exclusion must be regarded as a minimum. Table 6 shows that, after primary adsorption of inactivated NJ VS to varying multiplicity, the number of secondary serotype infective centres present in a monolayer after secondary adsorption of about 80 live heterotypic particles (1 per 2×10^5 cells) was significantly affected only when the average multiplicity of primary infection was between 1 and 5.

Comparison of these data with those of Baluda (1957), who showed that only one inactivated Newcastle disease virus particle was required to initiate exclusion, strongly suggests that many VS particles are required. This conclusion is supported by the large number of yielders (20–40% of total cells) usually found in cell populations completely infected with a high multiplicity of active or inactive interfering virus, despite a low (single) multiplicity of challenge virus. Since much heat-inactivated virus must also be present the process does not seem very efficient, but adsorption of the higher number of primary particles in Table 6 improved the efficiency, due perhaps to co-operation between particles, or an increased probability that a single particle may succeed.

Plaque size was also markedly reduced at about the inoculum size where most cells had received one inactivated particle, but it is noteworthy that plaques were still able to form when the multiplicity was 50. On these high multiplicity plates the cells between plaques stained well with neutral red and did not differ from controls in microscopical appearance. Thus inactivated virus alone did not harm cells, but in the plaques all cells were unstained and showed much cytopathic effect, so that the initial protection once afforded to at least half the cells had lapsed, either by the passage of time or by reversal by a high multiplicity of infection within the plaque. On the other hand, the fact that plaques were smaller means that interference must in many cells and in some form have lasted at least as long as the time required for infection of the second cycle in the plaques (4–12 hr.).

*Lack of effect of pre-inoculation with inactivated
homotypic VS virus on release curves*

Two monolayers were inoculated with a high multiplicity of UVI+storage-inactivated IND VS and a third with medium only as a control. After adsorption, one inactivated-inoculated plate and the control plate were completely infected with a low multiplicity (average=2) of active homotypic virus, and the other inactivated-inoculated plate had medium only as a second control. After a further adsorption period, the cells were removed with trypsin and

Table 5. *Decreasing efficiency of heterotypic live-virus exclusion by the primary inoculum with increasing multiplicity of superinfecting live virus*

Virus stock initially contained 1.2×10^8 pfu/ml. (IND) and 1.0×10^8 pfu/ml. (NJ), and had inactivated to 1.2×10^8 and 1.1×10^7 pfu/ml. respectively on storage at -20° . The primary inocula (0.5 ml.) were removed after 30 min., monolayers washed once and the secondary inocula (0.5 ml.) added for a further 30 min. The cells from each monolayer were then washed and resuspended in 1 ml. for assay. 'NJ serotype' and 'IND serotype' represent plaque assays with respectively IND and NJ antisera in the agar overlay; addition of both antisera to plaque assays from monolayer 5 gave no plaques. M=multiplicity of infection with live and 'stor'=storage-inactivated virus. $*=2 \times 10^{-4}$.																			
Infective centres per monolayer																			
Cells intact																			
Total pfu																			
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Virus stock initially contained 1.2×10^8 pfu/ml. (IND) and 1.0×10^8 pfu/ml. (NJ), and had and resuspended in 1 ml. for assay. 'NJ serotype' and 'IND serotype' represent plaque inactivated to 1.2×10^8 and 1.1×10^7 pfu/ml. respectively on storage at -20° . The primary assays with respectively IND and NJ antisera in the agar overlay; addition of both antisera inocula (0.5 ml.) were removed after 30 min., monolayers washed once and the secondary to plaque assays from monolayer 5 gave no plaques. M=multiplicity of infection with live inocula (0.5 ml.) added for a further 30 min. The cells from each monolayer were then washed and 'stor'=storage-inactivated virus. *= 2×10^{-4} .

Table 6. *Heterotypic interference in number and size of IND plaques by inactivated NJ VS*

The NJ stock originally contained 2×10^8 pfu/ml. and fell to 5×10^7 pfu/ml. at -20° , and was then ultra-violet irradiated to reduce survivors to a further 10^{-4} ; the average inactivated NJ particle behaved as if it had received 4 'storage' + 9 UV lethal damages. Varying dilutions of this stock were then adsorbed in 0.5 ml. amounts to 8 monolayers for 1 hr. at 37° , three further monolayers having medium only as control. The inoculum was removed, and 0.5 ml. of IND stock, diluted to contain about 80 pfu, was adsorbed at 37° for a further hr., when agar overlay containing NJ antiserum was added.

Monolayer no.	Primary inocula (NJ)		Secondary inocula (IND) Approx. pfu added	Plagues per monolayer		% IND-infected cells yielding progeny
	Diln. of stock	Multiplicity of total particles		> 2 mm. diam.	< 2 mm. diam.	
1	Neat	50	0	0	0	—
2	Neat	50	80	0	15	18
3	1/3	16	80	0	30	36
4	1/10	5	80	0	43	52
5	1/30	1.6	80	0	95	115
6	1/100	0.5	80	71	50	146
7	1/300	0.16	80	135	0	163
8	1/1000	0.05	80	79	0	95
9-11	Control	0	80	97, 74, 79	0	(100)

Table 7. *Lack of effect of pre-adsorption of storage-inactivated plus UVI IND VS on plating efficiency after subsequent infection with active IND VS*

The virus stock used initially contained 2×10^8 pfu/ml. and had inactivated to 1.7×10^8 pfu/ml. at -20° ; part of this stock was irradiated with UV to a survival of 3×10^{-3} (5 lethal hits). The primary inocula (0.5 ml.) were medium only or UVI stock diluted 1/2; after 1 hr. this was replaced by the secondary inocula (0.5 ml.) of medium only or active virus stock diluted

1/5. After a further 30 min. the cells were washed and resuspended to 1 ml. for assay. Part of this suspension was diluted in medium to 10^4 cells/ml. for 1-step virus release at 37° (Fig. 1). M = multiplicity of infection with UVI, storage-inactivated ('stor') or active virus.

Monolayer no.	Primary inocula M	Secondary inocula M	Total cells recovered $\times 10^6$	Infective centres per monolayer (pfu)			
				Total at 0.83×10^{-4}	per ml. $\times 10^4$	Plating efficiency (%)	Not sedimented by 3 min. at 1000 rev./min. at 10^{-3}
1	20 UVI + 50 'stor'	0	9.5	94, 87 (10^{-2})	1.8	0.19	1 (10^{-1})
2	0	2 active + 8 'stor'	9.25	116 131 131	302	32.4	16
3	20 UVI + 50 'stor'	2 active + 8 'stor'	9.75	95 102 107	242	24.6	10

suspended to 10^4 /ml. in a 50% (v/v) mixture of PBS and 'conditioned' medium for one-step virus release at 37° . Figure 1 shows that this pre-adsorption of inactivated VS had no effect on the release rate or latent period; a slight apparent decrease in final yield was not confirmed in other experiments and is not regarded as significant. As in Table 1 there was also no significant exclusion in terms of number of infective centres recovered (Table 7). In the suspension inoculated with inactivated virus only, the ratio of infective centres

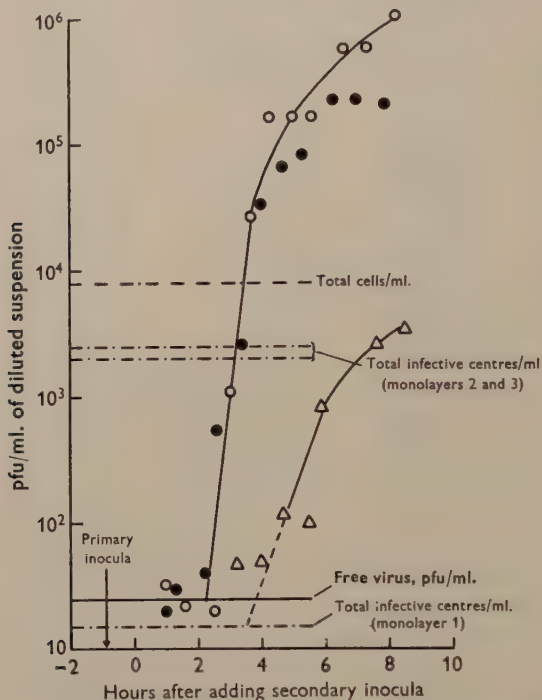


Fig. 1. Lack of effect of pre-adsorption of storage-inactivated plus UVI IND VS on 1-step virus release in suspension at 37° after subsequent infection with active IND VS. The data are from the same experiment as those of Table 7. Δ , monolayer 1; \circ , monolayer 2; \bullet , monolayer 3.

recovered to active particles added was if anything slightly less than the plating efficiency of the other cells, 13% compared with 30%, indicating that multiplicity reactivation was not occurring. The release curve of this suspension is less clear in the early stages than the others, but the latent period (3–5 hr.) is not very different from that usual for single infection (4.5 hr.) and the rate of release and final yield are also not much affected, despite the large excess of inactive virus adsorbed within the same 1 hr. period.

DISCUSSION

A problem raised by the phenomenon of shortened latency (Cooper, 1958), and the one dealt with in this paper, is whether or not each adsorbing particle of VS virus during more or less simultaneous multiple infection (i.e. during the

first half-hour of the latent period), contributes equally to the synthesis of progeny.

Interference and exclusion occur widely among viruses, and the precedent from bacteriophage work suggests that mutual exclusion may occur between unrelated viruses, but related viruses may allow some mixed infection. On the other hand, interference showing as cross-protection is a criterion of relationship among plant viruses. One cannot, therefore, predict from these cases whether exclusion might occur between virus particles of the same or different serotypes of VS virus.

One practical criterion for the problem stated above is the absence or presence of exclusion in the sense of the non-release, in infective form, of a particular genome participating in a mixed infection. Homotypic interference in the sense of depressed rate of release and lower yield by live virus (compared at varying multiplicities of infection) has been shown not to occur with dilute passage stocks of VS virus (Cooper, 1958), although undiluted passage stocks of both VS serotypes contain a non-infective component capable of marked homotypic exclusion (Cooper & Bellett, to be published). The present paper shows that UVI, heat- and 'storage'-inactivated dilute-passage VS virus preparations do not exclude live virus homotypically under conditions where the same inactivated preparations markedly exclude heterotypically. Live virus preparations also exclude heterotypically. The many observations (Luria, 1953) that practically no interfering phenomenon shown by live virus is destroyed by a relatively low number of UV lethal hits makes it seem likely that live VS virus will not exclude homotypically, so that all particles of a mixed infection should contribute equally to progeny. A more definite conclusion for VS virus must await further evidence, perhaps from genetically labelled strains.

Among the many cases of interference by animal viruses, the easiest interpretations, from a cellular viewpoint, involve influenza and Newcastle disease virus (NDV). Like VS, inactivated influenza virus interferes completely and rapidly with heterologous virus (except that only 1 particle may be required per cell (Fazekas de St Groth & Edney, 1952)), but not with homologous virus (save for some depression of yield), provided that super-infection occurs within a few hours (Frazer, 1953). On the other hand, Baluda (1957) showed that UVI NDV excluded homotypically and rapidly, and evidence was given to suggest that the content of non-infectious haemagglutinin was low. However, where 'incomplete' interfering virus may exist and where homotypic exclusion has been found in inactivated virus preparations (unlike dilute-passage VS), further evidence is required to ensure that such exclusion cannot be due to a relatively low content of non-infectious haemagglutinin, e.g. equal in number to infective particles. Baluda also found that, like heterotypic VS, the exclusion in homotypic NDV was to some extent reversed by an increase in multiplicity of the superinfecting virus, although the exclusion appeared much more efficient in the case of NDV in that one particle only was required, whereas VS (heterotypic) probably needs several or very many. VS (homotypic) is even less efficient in that no exclusion was detectable up to

multiplicities of 70; this contrasts markedly with the efficiency of the non-infectious component of VS undiluted passage (Cooper & Bellett, to be published), in which a small number of particles, perhaps one, excluded homotypically.

The presence of marked heterotypic exclusion in VS when the secondary inoculum was added only 12 min. after the primary suggests that the process of establishing exclusion may be quite rapid. It should be made clear, however, that this may not be the case. If the apparent critical time for exclusion of the secondary particle was, for example, 1 min. after the primary, this merely means that the process for irrevocably establishing the secondary infection is shorter by one minute than the process of irrevocably establishing exclusion. Only in the special case where the time for completely establishing the secondary infection is infinitely small can exclusion be said to be established in 1 min. The process could in fact be quite lengthy, and an apparently rapid exclusion indicates rather that the two processes of infection and exclusion are of similar length. The apparent reversibility of exclusion with increasing multiplicity of secondary infection (which may also explain the minority of cells which fail to show heterotypic exclusion) may be explicable in terms of a competition for a particular site or of a change akin to shortened latency in probability or rate of establishment either of infection or of exclusion which is due to the increase in multiplicity of infection.

I am grateful to the staff of the Research Institute, Pirbright, Surrey, for the original virus stocks, and for several generous gifts of high titre specific antisera.

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Formation of Enzymes Involved in Pyrimidine Synthesis and Amino Acid Metabolism in *Escherichia coli*

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SUMMARY: *Escherichia coli* B10, a mutant strain of *E. coli* B, which exhibited a requirement for histidine+uracil under certain conditions of growth, became pyrimidine-independent when grown in a simple medium supplemented with these two substances. It was demonstrated that this change from uracil-dependence to non-dependence was not due to the selection of a competent back-mutant, but to the formation of the enzyme dihydro-orotic acid dehydrogenase, which is lacking in uracil-requiring organisms. A study of the enzyme content of mutant and wild type *E. coli* at various stages of growth demonstrated that a high enzyme-forming capacity is associated with young organisms harvested from cultures in the late lag or early logarithmic period.

The growth of organisms is accompanied by qualitative and quantitative changes in their enzymic activities (Needham, 1942; Greenstein, 1947; Potter, 1950; Hardwick & Foster, 1953; Dolphin & Frieden, 1955). The enzyme composition appears to be dependent upon the age of the organisms (Gale, 1938; Rogers, 1954, 1957; Barnes & Morris, 1957; Holmes, 1955; Sheinin, 1956) the physical conditions of their growth (Gale & Epps, 1942; Gale, 1943; Rogers & Spensley, 1955; Holmes, 1955; Goebel, Barry & Shedlovsky, 1956; Giles, Partridge & Nelson, 1957), as well as upon the constituents of the growth medium (Monod, 1942; Nason, Kaplan & Oldewurtel, 1953; Spiegelman, 1950).

The results of investigation of induced enzyme formation (Cohn & Monod, 1953; Pollock, 1953), enzyme inhibition (Yates & Pardee, 1956*b*) and enzyme repression (Vogel, 1956, 1957; Yates & Pardee, 1957) suggest that any or all of these mechanisms may participate in moulding the enzyme pattern of organisms. Further study of this problem was made possible as a result of the finding, described in the present communication, that in the course of growth on uracil-containing medium an apparent uracil-requiring mutant of *Escherichia coli* acquired the ability to grow without an exogenous supply of pyrimidine.

METHODS

Organisms. Two organisms were used: (i) a laboratory strain of *Escherichia coli* B; (ii) a mutant of another strain of *E. coli* B, the mutant *Escherichia coli* B10, which requires for its growth histidine and, under certain conditions

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(to be discussed), uracil or cytosine. This organism was obtained from Dr S. Brenner of the Medical Research Council Unit of Molecular Biology, University of Cambridge.

Media. *Escherichia coli* B 10 was maintained on slopes of a medium containing (w/v): 1% glucose, 0.1% Marmite (Marmite Ltd., London), 3% tryptic hydrolysate of casein (Gale, 1947) and 2% agar (Harrington Bros. Ltd., London). This medium will be referred to throughout as the E medium. *Escherichia coli* B was similarly maintained on C medium of Roberts *et al.* (1955) supplemented with glucose (1%, w/v) and agar (2%, w/v). In all cases inoculated slopes were incubated for 24 hr. at 37° and then kept at room temperature for not longer than 1 week. These cultures were used to give inocula for experimental work.

In some instances large volumes of inoculum were required. To obtain these, organisms were cultured as above on solid medium in Roux bottles.

For growth experiments and enzyme analyses organisms were grown in liquid medium in volumes of 400 ml. in 1 l. Florence flasks, shaken in water baths at 37°. The basal medium, which will be referred to throughout as the C/G medium, was the C medium (Roberts *et al.* 1955) containing glucose (0.2%, w/v). The other media, the C/G/H and C/G/HU media, consisted of C/G medium supplemented with L-histidine (Roche Products Ltd., 10 µg./ml.) and uracil (Light's Chemical Co., 10 µg./ml.) as indicated by H and U respectively.

Growth was estimated by measuring the optical density of a culture at 420 mµ in a Beckmann DU spectrophotometer, unless otherwise indicated. It has been found (Sheinin, 1956) that the optical density of cultures of *Escherichia coli* B is directly proportional to the bacterial dry weight from early in the lag period of growth, throughout the logarithmic phase and into the early stationary phase.

Enzyme preparations. Enzyme activity was examined in extracts prepared in the following way. Organisms grown in liquid medium were harvested by centrifugation for 20 min. at 4000 rev./min. To harvest organisms grown on solid medium the growth on the surface of the agar was washed off with 10–15 ml. C medium and centrifuged. The organisms were washed with ice-cold distilled water and resuspended in a volume of the same, approximately equal to the volume of the deposited organisms.

The organisms were disrupted according to the conditions worked out by Dr R. Davies, using a transducer designed to be employed with the 25 kilocycle Mullard oscillator. Two ml. portions of the above bacterial suspension were added to the stainless steel cup. The outer jacket was kept packed with crushed ice so that the temperature of the suspension never rose above 5°–8°. The suspension was subjected to supersonic vibrations for 1 min.; this gave complete cell breakage. As viewed in the microscope, with phase contrast, the preparation consisted of large granules, debris and some ghosts; no intact organisms were detected.

The broken cell suspension (or combined suspension in cases where the volume of the original suspension was greater than 2.0 ml.) was centrifuged

at 2° for 5 min. at 4600 g. The supernatant fluid was removed and stored at -20° pending enzyme assay.

The nature of the supernatant fluid varied, depending upon the age of the organisms from which it was obtained. Young organisms, from the lag phase and logarithmic phase of growth, yielded supernatant fluids which were clear, slightly viscous and a pale yellowy-pink in colour. The supernatant fluids from stationary phase organisms, whether grown on liquid or solid medium were turbid, viscous and a definite pink in colour. No attempt was made to fractionate the supernatant fluids, which will be referred to throughout as 'the enzyme preparations'.

Enzyme assays. The ureidosuccinic acid (USA) synthetase (Jones, Spector & Lipmann, 1955), the dihydro-orotase (Lieberman & Kornberg, 1954) and the dihydro-orotic acid (DHO) dehydrogenase (Lieberman & Kornberg, 1953) were measured by methods based on those used by Yates & Pardee (1957).

(i) *Ureidosuccinic acid synthetase.* Enzyme preparation (0.2 ml.) was incubated in a small centrifuge tube at 37°, with 0.1 M-potassium phosphate buffer pH 6.2, 0.03 M-aspartic acid (pH of stock solution adjusted to 6.2 with potassium hydroxide) and 0.02 M-lithium carbamyl phosphate (Jones *et al.* 1955). The last was added as a fine suspension in water. To terminate the reaction 2.0 ml. of trichloroacetic acid (TCA, 5%, w/v) was added. After overnight storage at 2° this reaction mixture was centrifuged; the clear colourless supernatant fluid was filtered under suction and the TCA was removed by extraction with three 5 ml. portions of ether.

The concentration of USA in the extracted filtrate was assayed on 1.0 ml. portions by the procedure of Koritz & Cohen (1954) with slight modification. The diacetyl monoxime reagent was made up in 60% (v/v) ethanol in water and 0.5 ml. potassium persulphate solution was added in the final stage. The colour formed was measured at 555 m μ , at which wavelength the absorption curve for the USA-colour complex exhibits a maximum.

(ii) *Dihydro-orotase.* Enzyme preparation (0.2 ml.) was incubated as above with 0.07 M-sodium acetate buffer (pH 5.5) and 0.07 M-USA (pH of stock solution adjusted to 5.5 with sodium hydroxide). Enzyme activity was halted by adding 2.0 ml. of perchloric acid (PCA, 5%, w/v). The precipitated reaction mixture was stored overnight at 2°. A filtrate of the supernatant fluid was obtained as above.

To measure the amount of DHO in the filtrate a 1.5 ml. portion was incubated with 1.5 ml. M-sodium hydroxide at room temperature in a Beckman silica cuvette. The optical density at 240 m μ was measured at various time intervals. The initial rate of decrease in the ultraviolet absorption was found to bear a linear relationship to DHO concentration and was therefore used as a measure of this concentration.

(iii) *Dihydro-orotic acid dehydrogenase.* Enzyme preparation (0.2 ml.) was incubated as above with 0.1 M-potassium phosphate buffer (pH 7.88), 15 μ mole reduced diphosphopyridine nucleotide (Sigma Chemical Co.) and 15 μ mole orotic acid (Light's Chemical Co.). The dihydro-orotic acid formed was

measured as described in (ii) in filtered supernatant fluids of PCA-precipitated reaction mixtures.

(iv) *Aspartase and histidase*. To measure the aspartase and the histidase activities of the enzyme preparation, 0.1 ml. was incubated as above with 0.05M-potassium phosphate buffer (pH 7.0), 0.015M L-aspartic acid or L-histidine HCl (stock solutions made up in 0.05M-phosphate buffer). To terminate the reaction a 1.0 ml. portion of the reaction mixture was added to the outer compartment of a Conway vessel (Conway, 1947) containing 1.0 ml. of 40 % (w/v) potassium hydroxide. The vessels were incubated at 37° for at least 90 min. to permit the ammonia released to distil over into the 2.0 ml. of N-hydrochloric acid in the centre well. One ml. portions of the ammonium chloride solution so formed were assayed for their nitrogen content by the procedure of Johnson (1941).

All measurements of enzyme activity were made in duplicate in reaction mixtures of a total volume of 1.2–1.5 ml., under conditions and for periods of time during which enzyme activity was linear. The enzyme activities are expressed throughout as μ mole product formed/hr./mg. protein (measured by the method of Lowry, Roseborough, Farr & Randall, 1951) in the enzyme preparation. Bovine plasma albumin (Armour Laboratories) was used as the standard protein.

RESULTS

Uracil requirement of Escherichia coli mutant B10

Escherichia coli B10 grown on solid E medium and then subcultured into a simple ammonia salts medium (C medium) exhibited an absolute requirement for histidine + uracil. Even after incubation for as long as 48 hr. at 37° the organism did not grow without uracil. However, when cultured in a fully supplemented medium (C/G/HU) *E. coli* B10 acquired the ability to grow subsequently in the absence of added uracil (Fig. 1). Organisms harvested from an E medium slope were grown in C/G/HU medium at 37° (curve 2). At various times portions of the culture were centrifuged, the organisms washed once with a large volume of C medium and suspended in the same to a constant optical density. Such suspensions of organisms were used to inoculate standard volumes of C/G/H medium. These cultures were shaken at 37° for 24 hr. and their optical density (at 420 m μ) was measured (curve 3).

Little or no change in the optical density of C/G/H cultures, inoculated with organisms from very young or very old cultures made in C/G/HU, was observed. However, when C/G/H cultures were sown with organisms harvested from C/G/HU cultures in the logarithmic and early stationary phases, these exhibited different increases in optical density after 24 hr. It seems reasonable to conclude that the observed increases in optical density of all the cultures represent growth of the organism. Although no bacterial counts were made in these experiments, there is little doubt that the large changes in optical density were due to increases in numbers of organisms. However, it is possible that the small change in optical density observed when inocula from the late lag, early exponential and very late stationary phase

were employed, was due not to multiplication but to a change in shape or size of the existing organisms brought about by growth of cellular constituents.

Thus it was concluded that as the *Escherichia coli* B10 cells grew in the C/G/HU medium, they lost their uracil dependence. The ability to grow subsequently without uracil (curve 3) increased as the culture passed through the logarithmic period (curve 2), reached a peak, and then declined as the original culture entered the stationary phase of growth.

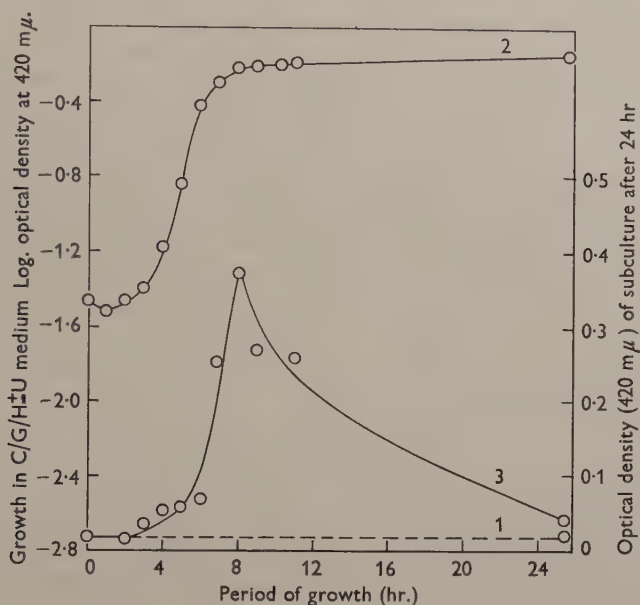


Fig. 1. Growth of *Escherichia coli* B10 in defined medium in the absence of uracil. Curve 1: growth of organisms subcultured from E medium slopes into C medium supplemented with glucose (0.2%, w/v) and histidine (10 μ g./ml.). Curve 2: growth of organisms cultured as in curve 1 in C/G/H medium containing added uracil (10 μ g./ml.). Curve 3: optical density after 24 hr. of subcultures made in C/G/H medium of organisms harvested from the C/G/HU culture, the growth of which is shown in curve 2.

This phenomenon was not dependent upon the size of the inoculum used. The same kind of response (with some quantitative variations) was obtained whether a very large or a very small inoculum was used. In all cases the ability to grow without uracil was acquired by organisms in the course of their growth in the C/G/HU medium, and usually appeared early in the growth cycle.

This change from dependence on, to independence of, exogenous uracil suggested that in the course of their growth on a uracil-containing medium, the organisms acquired one or more enzymes which participated in the synthesis of pyrimidines. Before going on to study this phenomenon it was essential to be certain that it was not due to the selection of a back mutant, but was in fact associated with the growth of the original *Escherichia coli* B10. The hypothesis that the above experimental findings were due to selection of

a back mutant able to synthesize uracil would seem to be excluded by a number of facts.

(a) The ability to grow without uracil, once acquired by the organisms, is not a stable characteristic, but is lost as the organisms continue to grow (Fig. 1).

(b) If competent back mutants had appeared during growth on the E medium agar, one would hope to provide them with a selective advantage for growth by inoculating C/G/HU medium with inocula of increasing size. However, in no instance was growth obtained, even after incubation for 48 hr., with inocula containing up to the order of 10^{10} – 10^{12} organisms.

(c) That the selection of back mutants appearing in the course of growth in the C/G/HU medium seemed unlikely to explain the results was indicated by the data obtained in Fig. 1. Assuming back mutants had appeared one might postulate the following. (i) The growth rate of the mutant might be equal to that of *Escherichia coli* B10; in this case the same number of mutant organisms should be present in each inoculum and therefore the 24 hr. optical density achieved in C/G/H medium should be the same irrespective of the age of the inoculum. (ii) The growth rate of the mutant might exceed that of the parent; in this case an increasing proportion of the inoculum should be mutant organisms, and again the 24 hr. culture density should be the same, being limited only by the maximum growth-supporting capacity of the medium and the growth characteristics of the organisms (Monod, 1949). (iii) The growth rate of the mutant might be less than that of the parent; in this case the maximum ability for growth without uracil should be observed at the time at which the mutant appears, and should decline thereafter. Clearly none of the postulates is in accord with the experimental findings.

One further attempt was made to test for a uracil-synthesizing back mutant. Organisms similar to those used to obtain curve 3, Fig. 1, were plated on C/G/H medium solidified with 2% (w/v) agar. If the onset of uracil independence were associated with the appearance of a back mutant, and this mutant occurred at the rate at which most mutations in *Escherichia coli* have been found to occur (Lederberg, 1949), then by plating 10^5 to 10^7 organisms one would hope to pick up from one to 100 colonies/plate. Organisms harvested during the lag phase of growth in C/G/HU medium, which were unable to grow in C/G/H medium, yielded no colonies in the plating test (Table 1). However, organisms harvested once growth in the uracil-containing medium had begun, were capable of growth in non-uracil medium and gave rise to confluent growth on the plates. This result indicated that all, or certainly a majority, of the organisms plated were capable of growth without uracil, and that therefore the observed uracil-independent growth was not due to the selection of pyrimidine-synthesizing back mutants.

Uracil utilization by Escherichia coli mutant B10

A second possible explanation for the growth of *Escherichia coli* B10 in uracil-free medium is that in the course of their growth in the C/G/HU medium, the organisms take up and store, in a free or bound form, sufficient

uracil to satisfy their needs when transferred to C/G/H medium. This hypothesis seems improbable in view of the small number of organisms which were subcultured and which subsequently gave rise to cultures of approximately 1 mg. dry wt. organisms/ml., therefore containing of the order of 23 μ g. pyrimidine/ml. (Roberts *et al.* 1955). To examine this possibility further the uracil utilization by *E. coli* B10 was followed. Organisms were incubated as

Table 1. *Subsequent growth of Escherichia coli B10 previously grown in C/G/HU medium*

Washed organisms, harvested from E medium slopes, were grown in C/G/HU medium. After varying periods of incubation organisms were subcultured into liquid C/G/H medium and on to C/G/H agar plates; growth was assessed by optical density and colony counts respectively.

Duration of growth in C/G/HU (hr.)	Optical density of culture (420 m μ)	24 hr growth of subculture in C/G/H	Colony growth on C/G/H agar
0	0.006	None	None
1	0.006	None	None
4	0.006	None	None
7	0.057	+	Confluent growth
9	0.394	+++	Confluent growth

in curve 2, Fig. 1. At different times portions of the culture containing the equivalent of 1–10 mg. dry wt. organisms were chilled and centrifuged at 20,000 *g* at 2° for 15 min. The optical density at 260 m μ of the culture supernatant fluid was taken as an index of the uracil content. The pellet of organisms was extracted first with 5 ml. 5 % (w/v) perchloric acid at 2° for 2 hr., and then with 5 ml. 5 % (w/v) perchloric acid at 70° for 30 min. The u.v. absorption of the extracts was taken as a measure of the amount of free purines + free pyrimidines + nucleotides in the first case, and of the nucleic acid content of the organisms in the second. The results obtained are shown in Fig. 2.

No detectable disappearance of uracil (Fig. 2, curve *b*) occurred until growth had begun (Fig. 2, curve *a*). The decrease in uracil content of the medium appeared to be directly related to the growth of the organism. The study of uracil utilization beyond 3 hr. was obscured by the appearance in the medium of some u.v. absorbing material. However, it is clear from curve *c* that the organisms did not store large amounts of uracil either in free or nucleotide form. If the organisms were enabled to grow in uracil-free medium as a result of their storage of pyrimidine, then one would have expected that the concentration of cold perchloric acid-soluble substances should increase as the organism grows in C/G/HU medium; whereas in fact the amount of such materials decreased exponentially throughout the growth period. Similarly, curve *d* indicates that there was no tendency for the storage of uracil as nucleic acid.

Enzyme activity of Escherichia coli mutant B10

To assess the biochemical nature of the change from uracil dependence to independence a study was made of the enzyme constitution of the organisms. The enzymes investigated were the ureidosuccinic acid synthetase (USA), the

dihydro-orotase and the dihydro-orotic acid (DHO) dehydrogenase, which are known to participate in pyrimidine synthesis in *E. coli* (Yates & Pardee, 1956*a*). In addition, in view of their possible involvement under the present experimental conditions, the aspartase (Gale, 1938) and histidase (Leuthardt, 1950) activities of the organisms were also examined.

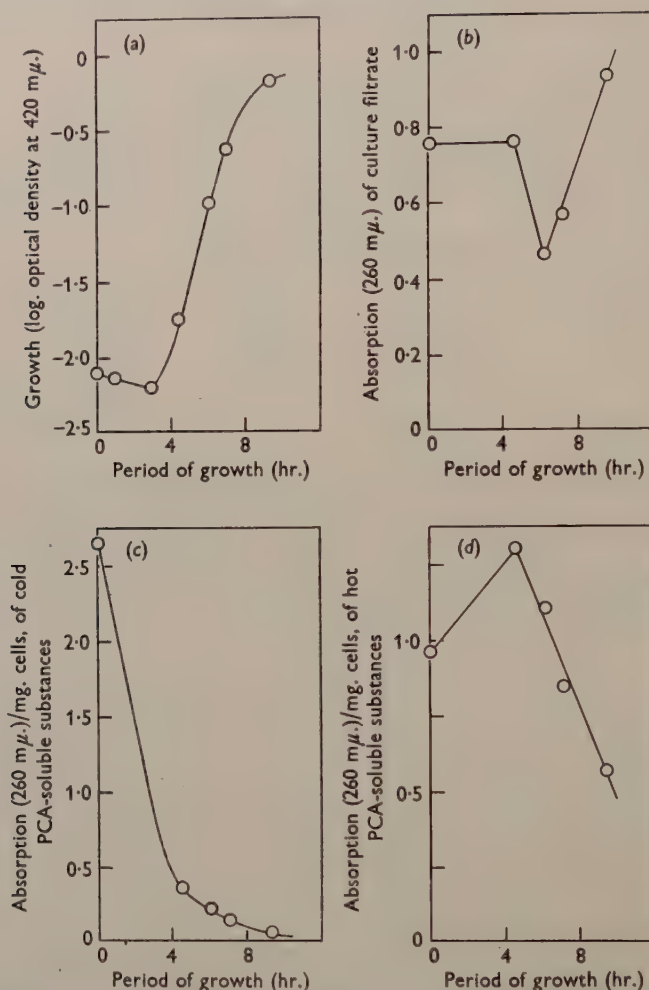


Fig. 2. Utilization of uracil by *Escherichia coli* B10. (a) Growth of organisms in C/G/HU medium; (b) ultraviolet absorption of culture filtrate; (c) ultraviolet absorption of the cold PCA-soluble substances in *E. coli* B10; (d) ultraviolet absorption of the hot PCA-soluble substance in *E. coli* B10.

The activity of the amino acid deaminases, the USA synthetase and the dihydro-orotase, although low in the inoculum, rose to reach a maximum early in the logarithmic phase of growth, and then declined (Fig. 3). The *E. coli* B10 organisms which were initially devoid of DHO dehydrogenase activity, acquired this activity during growth in the C/G/HU medium (Fig. 3*a*). Since the detection of DHO dehydrogenase activity depends on the

availability of orotic acid it was possible that the absence of activity from the organisms of the inoculum might be due to the rapid removal of the substrate by some other metabolic pathway. This explanation was excluded by two findings: (i) under the test conditions used there was little or no orotic acid

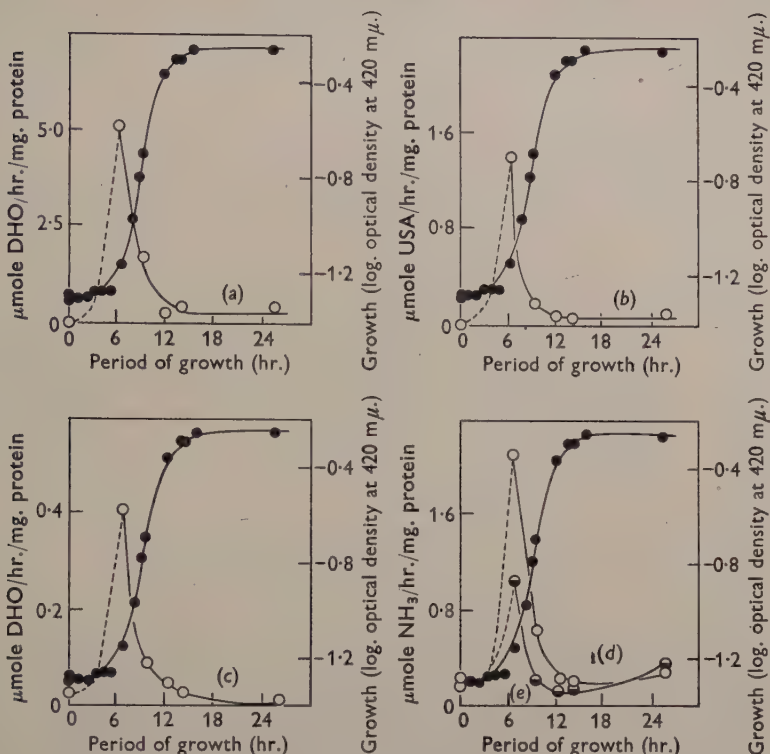


Fig. 3. Enzyme formation in *Escherichia coli* B10 grown in C/G/HU medium. ●—●, growth; ○—○, —●—●, enzyme activity. (a) Dihydro-otric acid dehydrogenase; (b) ureidosuccinic acid synthetase; (c) dihydro-otricase; (d) aspartase; (e) histidase.

decarboxylase activity; (ii) in the absence of added reduced diphosphopyridine nucleotide, the optical density of the perchloric acid-extract of the reaction mixture remained essentially unchanged, indicating that the orotic acid remained unutilized. Thus the inability to detect DHO dehydrogenase activity in the inoculum does reflect an absence of such enzyme activity in the enzyme preparation.

To determine the relationship between the appearance of DHO dehydrogenase activity and the loss of uracil dependency, a closer examination was made of organisms in the lag period, with the results shown in Fig. 4. Those organisms which could not grow when subcultured into C/G/H medium exhibited no detectable dehydrogenase activity; whereas organisms able to grow without added uracil did possess measurable dehydrogenase activity. The activity of the other enzymes studied, low throughout the early lag period, increased as the organisms began to grow in the C/G/HU medium.

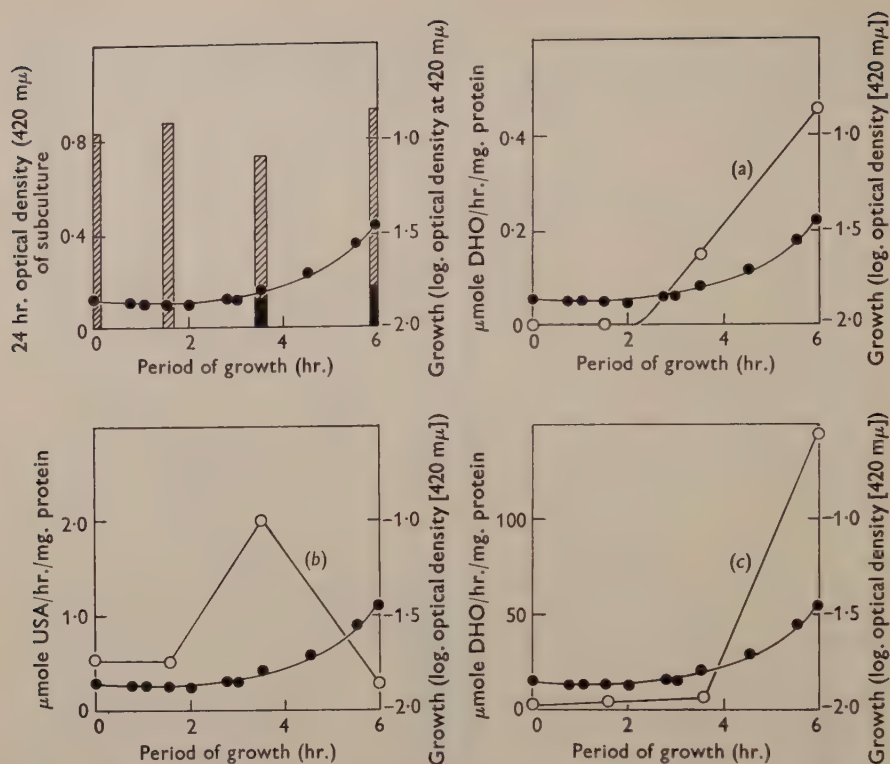


Fig. 4. The relationship between the enzyme activity of *Escherichia coli* B10 grown in C/G/HU medium and their capacity for growth without added uracil. The cross-hatched bar graphs represent subsequent 24 hr. growth in C/G/HU medium, and the dark bar graphs subsequent 24 hr. growth in C/G/H medium, of organisms harvested from a C/G/HU culture, the growth of which is shown in curves ●—●. Enzyme activity is denoted by the curves ○—○; (a) dihydro-otic acid dehydrogenase; (b) ureidosuccinic acid synthetase; (c) dihydro-ototase.

Table 2. Comparison of the enzyme activities of *Escherichia coli* B grown with and without uracil

Washed organisms harvested from E medium slopes, were subcultured into C/G/H medium with (+) and without (−) added uracil. Enzyme activities are expressed as μmoles product formed/hr./mg. protein.

Phase of growth at which organisms were harvested	Aspartase		Histidase		Ureido-succinic acid synthetase		Dihydro-ototase		Dihydro-otot acid dehydro-genase	
	−	+	−	+	−	+	−	+	−	+
Early exponential	6.28	11.4	2.19	13.6	1.21	0.63	3.11	4.08	0.68	13.6
Mid exponential	6.55	26.7	6.79	12.6	0.46	0.34	7.69	12.0	0.35	0.90
Late exponential	3.89	7.8	1.28	2.80	0.09	0.07	2.27	0.32	0.09	0.32
Early stationary	0.77	12.8	2.65	2.70	0.04	0.04	3.37	0.21	0.18	0.53
Late stationary	0.38	14.1	4.53	6.10	0.02	0.09	2.32	0.21	0.14	0.54

Enzyme activity of Escherichia coli B

In order to be certain that the absence of DHO dehydrogenase activity from the *Escherichia coli* mutant B10 organisms was not an artifact, it was essential to know whether DHO dehydrogenase activity could be expressed under the experimental conditions employed. To test this the above experiment was repeated with the wild type *E. coli* B (Fig. 5). It is clear that

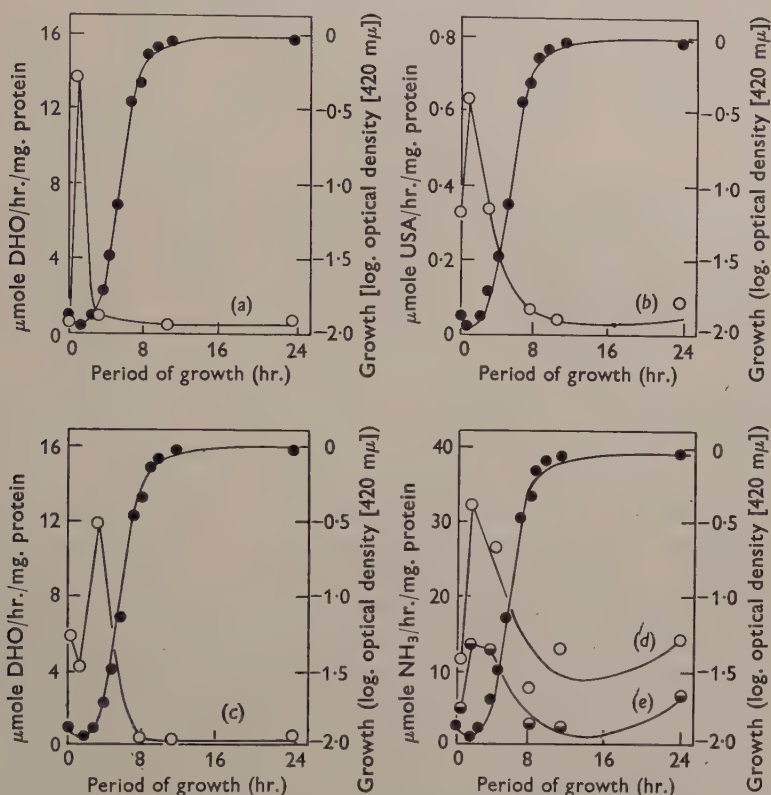


Fig. 5. Enzyme formation in *Escherichia coli* B grown in C/G/HU medium. ●—●, growth; ○—○, —●—●, enzyme activity; (a) dihydro-ototic acid dehydrogenase; (b) ureido-succinic acid synthetase; (c) dihydro-ototase; (d) aspartase; (e) histidase.

growth of the *E. coli* B on E medium agar did not result in the complete suppression of its DHO dehydrogenase activity. As in the case of *E. coli* mutant B10 the enzyme pattern of *E. coli* B organisms differed at different times during growth. Qualitatively similar results were obtained when *E. coli* B was grown with or without added uracil, although quantitative differences were observed (Table 2).

Test for inhibitor of dihydro-ototic acid dehydrogenase activity

To test whether the absence of DHO dehydrogenase activity from some *Escherichia coli* B10 extracts was due to the absence of active enzyme or to

the presence of an enzyme inhibitor, the activity of an active preparation was measured in the presence of an inactive extract; this did not alter its activity (Table 3). Thus it is clear that an inhibitor of DHO dehydrogenase activity was not present in the inactive *E. coli* B10 extract. In addition the active preparation does not appear to contain a dissociable activator or cofactor capable of influencing the inactive extract.

Table 3. *Dihydro-orotic acid dehydrogenase activity of extracts of Escherichia coli*

Aqueous extracts of *E. coli* B and *E. coli* B10 grown in C/G/HU were obtained from organisms disrupted by ultrasonic vibrations.

Extract used	Volume of extract (ml.)	Dihydro-orotic acid dehydrogenase activity (μ mole dihydro-orotic acid/hr.)
<i>E. coli</i> B10	0.10	0.000
<i>E. coli</i> B	0.10	0.151
<i>E. coli</i> B + } <i>E. coli</i> B10 }	0.10 ml. of each	0.157

DISCUSSION

Initially unable to grow without an exogenous supply of pyrimidine, *Escherichia coli* mutant B10 acquires the ability to do so when grown with uracil. In undergoing this change from uracil requirement to non-requirement, the organisms, which were devoid of dihydro-orotic acid dehydrogenase activity, acquire the capacity for converting dihydro-orotic acid into orotic acid. The finding (Table 3) that the absence of dihydro-orotic acid dehydrogenase activity in the inactive *E. coli* B10 extracts is not due to the presence of an inhibitor or the lack of an activator, makes it likely that the appearance of dehydrogenase activity in the organisms in the course of growth is due to the formation of active enzyme protein. The limitation of the assay procedure makes it difficult to conclude whether the change from non-active to active extract is qualitative, or whether it is a quantitative change from an extremely low, unmeasurable, amount of dihydro-orotic acid dehydrogenase in the former instance. However, whether the enzyme is lacking, or is present in such small quantity as to make it inadequate to satisfy the multiple pyrimidine requirements of the organism, the result is the same, namely, failure to grow and multiply unless there is a ready source of preformed pyrimidine. Once provided with this, the organisms can synthesize the nucleic acid-containing machinery which appears to be essential for enzyme formation (Gale, 1955), and then proceed to form not only the missing dihydro-orotic acid dehydrogenase, but also other enzyme proteins.

It seems clear that the capacity for pyrimidine synthesis is not the only factor which controls the growth of *Escherichia coli* B10. For although the ability to grow without added uracil appears to be dependent upon the presence in the organisms of active dihydro-orotic acid dehydrogenase (Fig. 4), there is not a direct correlation between the cellular concentration of dehydro-

genase and the maximum growth achieved in non-uracil medium. It is perhaps not pyrimidine synthesis as such, but the formation of some pyrimidine co-factor or metabolic intermediate, which is the ultimate controlling factor in the growth of *E. coli* B10 in the absence of added uracil.

A problem as yet unsolved, but emphasized by many investigations (Gale, 1943; Pollock, 1953; Vogel, 1957) is raised once more by the present studies; viz. what is it that stimulates, directs and controls the formation of the constitutive enzymes and the low basal level of the inducible enzymes, which normally comprise the metabolic and developmental machinery of a living cell. Although hypotheses involving enzyme repression (Vogel, 1956, 1957) may be invoked to explain the *de novo* formation of dihydro-orotic acid dehydrogenase these must await further experimental study.

However, one fact emerges from this investigation: uracil does not repress the formation of the pyrimidine-synthesizing enzymes in *Escherichia coli* B or in *E. coli* B10 grown on a simple medium. On the contrary enzyme formation in these organisms rises sharply in the course of their growth into the exponential phase. The general decrease in specific enzyme activity which occurs in the late logarithmic period may reflect a cessation of enzyme formation and/or an instability of enzyme protein brought about by factors associated with depletion of the medium originally capable of supplying the uracil, histidine and glucose requirements for a culture of maximum population equivalent to 1 mg./ml. dry weight organism. This explanation is supported by the observation of Gale (1938), that whereas the specific aspartase activity of *E. coli* grown on a rich medium rose to reach a plateau maximum, the specific aspartase activity of organisms grown on a simple medium increased throughout the exponential period of growth to reach a peak, and then declined sharply during the stationary phase.

The absence of suppression of enzyme formation by uracil seems to be at variance with the observations made by Yates & Pardee (1957). However, these authors failed to take into account the different rates of growth of the mutant organisms studied, on media either lacking or supplemented with uracil or its precursors. If the specific enzyme activity of these organisms varied with growth in a manner similar to that observed with *Escherichia coli* B and *E. coli* B10, then it is likely that the different rates of growth obtained under their experimental conditions would be sufficient to account for the marked differences in enzyme content of the organisms. This difference is not due primarily to specific inhibition of enzyme formation by uracil, but appears to be a secondary phenomenon dependent upon growth.

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The Effect of Sudden Chilling on *Escherichia coli*

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SUMMARY: As found by earlier workers, *Escherichia coli*, growing in broth at 37° was rendered incapable of gross multiplication either on nutrient agar or in nutrient broth by sudden cooling in many diluents at 4°. Killing is due to the joint action of a suitable diluent and of sudden chilling, since survival was complete either after sudden chilling in 0.3M-sucrose or after gradual cooling in a potentially lethal diluent, such as Ringer's solution. Organisms in the stationary phase of growth were completely resistant. The susceptibility of growing organisms to sudden chilling changed rapidly during the exponential phase. Comparison with the survival after exposure to streptomycin, another bactericidal agent which has no effect on stationary phase cultures, showed that survival after chilling was not due to a fraction of the population being in the stationary phase.

Sudden cooling of *Escherichia coli*, strain B, infected with phage T2, had the same effect as ultrasonic disruption; namely, destruction of infective centres in the first half of the latent period followed, in the second half, by release of intracellular mature phage.

When a broth culture of *Escherichia coli* growing exponentially (i.e. logarithmically) at 37° is suddenly added to a suitable diluent at 4°, the majority of the bacteria are subsequently unable to form colonies on nutrient agar. A stationary phase culture is unaffected by this treatment (Sherman & Cameron, 1934). Apart from the report by Hegarty & Weeks (1940), who described cyclical fluctuations in susceptibility during the exponential phase of growth which were attributed to partial synchronization of division, no other investigations of this phenomenon have been traced. The present paper describes means by which survival can be either increased or decreased; the conditions under which spontaneous changes in resistance occur and their relation to other examples of phenotypic resistance to antibacterial agents; and the use of sudden chilling for inducing premature lysis of phage-infected bacteria.

METHODS

Organisms. A prototrophic strain of *Escherichia coli* isolated from infected urine was used in all experiments save those using phage-infected bacteria, where *E. coli*, strain B, infected with phage T2, was used.

Media. Liquid media were either broth containing 0.5% (w/v) Difco yeast extract and 2% (w/v) Difco Bacto Casamino Acids Technical in distilled water, or the glucose + salts medium described by Hershey (1955) with

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KH_2PO_4 replaced by 1.52 % (w/v) sodium glycerophosphate, the final pH in either medium being 7.2–7.4. All liquid cultures were incubated in a water-bath at 37° and were aerated by bubbling. Foaming of broth was prevented by adding a trace of Silicone Anti-Foam M 432 (I.C.I., Gloucester House, 149 Park Lane, London, W. 1) with the tip of a straight wire. This antifoam can be sterilized by autoclaving and should the emulsion break, it can be re-established by vigorous agitation. Oxoid Nutrient Broth No. 2 (Oxo Ltd., 16 Southwark Bridge Road, London, S.E. 1), solidified with 1.5 % (w/v) New Zealand agar, was used for solid medium, any additions being made after the medium had been melted and cooled to 50°.

Counts. All colony counts were made by spreading samples of 0.2 ml. from successive tenfold dilutions on well-dried plates, one plate being inoculated from each dilution. Each point was thus derived from a count of up to 300 colonies. Viable counts of unchilled samples were made by dilution in full-strength Ringer's solution at room temperature, while those of chilled samples were made in the diluent used for chilling. The term 'survival' refers to the fraction, colony count after chilling/colony count on nutrient agar before chilling; while the term 'efficiency of plating on medium A' is the fraction, colony count on A/colony count on nutrient agar. The rate of growth of normal cultures was also estimated by means of a nephelometer (Evans Electro-selenium Ltd., Harlow, Essex). Conventional techniques were used in the experiments with phage T2 (Adams, 1950).

Chilling. Bottles containing 50 or 100 ml. diluent were placed either next to or in the freezing chamber of a domestic refrigerator in order to cool the diluent to +4° or –5°, respectively. Viable counts on chilled samples were done 3–4 hr. after chilling which allowed ample time for survival to become approximately constant.

RESULTS

Experiments with normal bacteria

Degree of survival after chilling. The upper part of Fig. 1 shows the growth curve of a broth culture at 37°, increasing with minimum doubling time of 20 min., while the lower part shows the survival on nutrient agar and on deoxycholate agar 4 hr. after dilution 1/100 in quarter-strength Ringer's solution at 4°. The inoculum came from a culture grown overnight at 37° and Fig. 1 shows that these organisms, which were presumably in the stationary phase of growth, and also those chilled after 4.5 hr. incubation, were unharmed by sudden chilling. However, in the middle of the exponential phase at 2 hr. after inoculation, survival was less than 2×10^{-4} on nutrient agar. Colony counts did not differ significantly from counts made in broth by the dilution method. On the other hand, as found by Sherman & Cameron (1934), no killing occurred when organisms in the exponential phase were cooled slowly by allowing 30 min. for the temperature to fall from 37° to 4°.

The organisms which survived sudden chilling nevertheless showed signs of damage since their efficiency of plating (see Methods) on nutrient agar containing cationic detergents was considerably less than that of either unchilled

or slowly chilled organisms (Figs. 1, 2); i.e. these detergents reduced the colony count of organisms surviving chilling far more than that of either unchilled or slowly chilled organisms. Similar values were obtained with survivors plated on nutrient agar containing either 0.25 % (w/v) sodium deoxycholate or 1/40 (v/v) 'Teepol L' (sodium or potassium salts of alkyl sulphates supplied by Shell Chemicals Ltd., 105/109 Strand, Norman House, London, W.C. 2),

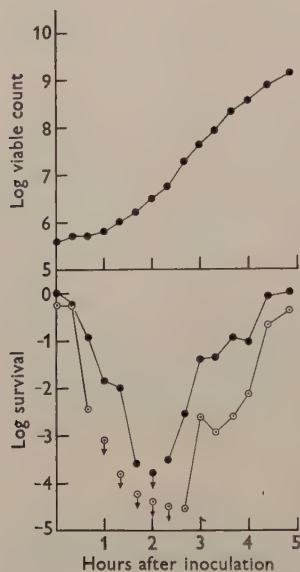


Fig. 1

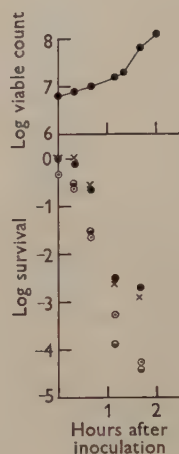


Fig. 2

Fig. 1. An overnight broth culture was diluted 1/2000 in fresh broth at 37°. Samples of 0.5 ml. were then diluted 1/100 in quarter-strength Ringer's solution at 4°, viable counts being made 4 hr. later on either nutrient agar (●—●) or nutrient agar containing 0.25 % (w/v) sodium deoxycholate (○—○). Points bearing arrows are derived from counts on samples in which no organisms survived to form colonies: each point gives the survival that would have been observed if one colony had been formed.

Fig. 2. The effect of various surface-active agents on survival of organisms chilled for 4 hr. in quarter-strength Ringer's solution at 4°. Counts were made on nutrient agar (●—●), and on nutrient agar containing either Tween 80 (x—x), sodium deoxycholate (○—○) or Teepol (○—○).

minimum efficiencies of plating being 10^{-2} – 10^{-3} , whereas the minimum efficiency for unchilled cells was 0.5–0.1. Neither the non-ionic detergent, Tween 80 (0.5 %, w/v: Honeywill and Stein, 21 St James St., London, S.W. 1) nor the anionic detergents, cetyl trimethyl ammonium bromide (≤ 0.1 %, w/v) or benzalkonium chloride (≤ 0.02 %, w/v) had any effect. The anionic detergents may have been inactivated by the medium since a heavy precipitate formed with the higher concentrations.

Organisms multiplying exponentially in glucose + salts medium at 37° with doubling-time of 1 hr. were far more resistant to sudden chilling in distilled water, the minimum survival after 4 hr. being 0.5 on nutrient agar and 0.1 on the same medium containing 0.25 % (w/v) sodium deoxycholate. The effect of

adding peptone (final concentration of 0.1–1 %, w/v) to exponentially increasing cultures in glucose+salts medium was also measured. The growth rate did not change for *c.* 35 min. after the peptone was added and during this period, the cells became completely resistant to chilling. The growth rate then increased and, concurrently, resistance to chilling declined, reaching a minimum *c.* 1.5 hr. later. A similar experiment was done using a mixture of prototrophic and auxotrophic organisms distinguished by their differing ability to

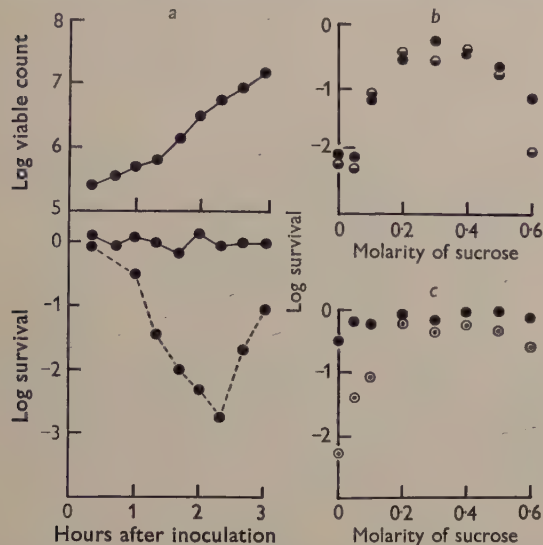


Fig. 3

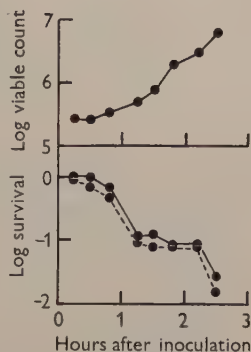


Fig. 4

Fig. 3*a*. Survival on nutrient agar after 4 hr. chilling in either quarter-strength Ringer's solution (●- -●) or 0.3*M*-sucrose (●-●).

Fig. 3*b* and *c*. Survival of 2 samples from growing broth cultures after 4 hr. chilling in various concentrations of sucrose at 4°. Counts were made on nutrient agar (●) and on nutrient agar containing either 0.25 % (w/v) sodium deoxycholate (○) or 0.3 *M*-sucrose (●).

Fig. 4. Survival on nutrient agar 4 hr. after sudden chilling either (*a*) by dilution 1/100 in distilled water at 4° (●-●), or (*b*) by dilution 1/2 in broth supercooled to -5°, the mixture (at a temperature of +6°–8° immediately after mixing) being transferred to a water-bath at +4° (●- -●).

ferment lactose and differing resistance to streptomycin. The susceptibility of both strains to chilling increased at virtually the same rate after the addition of peptone.

The influence of the diluent on survival after chilling. Susceptible organisms were completely unharmed by sudden chilling in sucrose, 0.3 *M* being optimal (Fig. 3). Partial protection was usually given by 10–16 % (w/v) propylene glycol 4000 (range of molecular weight 3300–3600 supplied by Oxirane Ltd., 170 Piccadilly, London, W. 1) dissolved in 0.1 *M*-Sørensen buffer at pH 6.8; by full strength Ringer's solution; and occasionally by broth. Sudden cooling of an exponential-phase culture by addition to an equal volume of broth supercooled to -5° was found to give the same survival as dilution 1/100 in distilled water at 4° (Fig. 4).

The appearance of chilled cultures. No morphological differences were found between formolized samples of unchilled cultures and formolized samples of cultures in which a majority of organisms had been killed by addition to an equal volume of saline at -5° . The total count determined by phase-contrast microscopy using a Helber chamber was essentially unchanged (Table 1); no differences in turbidity were seen by naked eye; no differences were detected by either phase-contrast or dark-ground microscopy of individual organisms or by electron microscopy of samples 2-4 described in the table.

Table 1. *Comparison of total and viable counts on chilled cultures*

Sample	Colony count/ml. on nutrient agar		Survival	Total count		Ratio: Total counts after/before chilling
	Before chilling	After chilling		Before chilling	After chilling	
1	3.1×10^7	4.56×10^6	0.15	2.42×10^7	2.17×10^7	0.90
2	3.7×10^7	4.5×10^6	0.12	5.76×10^7	5.13×10^7	0.89
3	5×10^7	6.5×10^6	0.13	4.88×10^7	4.44×10^7	0.91
4	8×10^7	2.75×10^7	0.34	8.05×10^7	8.67×10^7	1.08

Samples were chilled by addition to an equal volume of saline supercooled to -5° , the mixtures being at once transferred to a water bath at 4° for 4 hr. After a viable count had been made, formalin was added to 0.5 %, a portion taken for a total count, and the remaining suspension centrifuged, the deposit being resuspended in 1/10 vol. 0.5 % formalin for examination by electron microscopy. *Unchilled samples* were mixed with an equal volume of 1 % formalin at 20° and 4 hr. later, after a total count had been made, were concentrated in the same way.

The significance of phenotypic resistance to sudden chilling. When stationary-phase organisms were added to fresh broth, susceptibility to chilling usually increased after 20 min. (Fig. 1). However, maximum susceptibility did not develop until 1.6 hr. later. Similarly, susceptibility usually began to fall when the viable count reached 5×10^7 /ml., that is, about 1 hr. before any fall in the rate of increase of the culture was detectable by either colony counts or nephelometry. If the initial count was too great (say, more than 5×10^7 /ml.) there was too little time for maximum susceptibility to develop as the early phase of increasing susceptibility merged with the later phase when susceptibility fell.

Susceptibility presumably develops gradually because the organisms of the stationary phase inoculum differ in the time taken to become sensitive to chilling. In the second half of the growth curve, susceptibility presumably falls because, as the concentration of organisms increases, the mean rate of growth of the culture falls, leading to a change in survival. The survivors might have comprised a non-multiplying fraction of the culture, but this possibility was excluded by comparing the survival after chilling with that following exposure to a relatively high concentration of streptomycin, another agent which kills rapidly dividing organisms but which has no effect on organisms in the stationary phase of growth (Garrod, 1948; Mitchison & Selkon, 1956). If a fraction of the bacterial population was non-dividing, the ultimate survival after either chilling or exposure to streptomycin should be

the same. Figure 5 shows the effect of streptomycin on samples taken at various times in the second half of the growth curve. Curves *A* and *B* do not differ essentially; *C* shows a resistant fraction which will be considered in the Discussion; and *D* has the same form as *A* and *B*. The next six curves show a progressive increase in the resistance of the culture until the population is finally completely resistant. Comparison of Figs. 1 and 5 shows that when

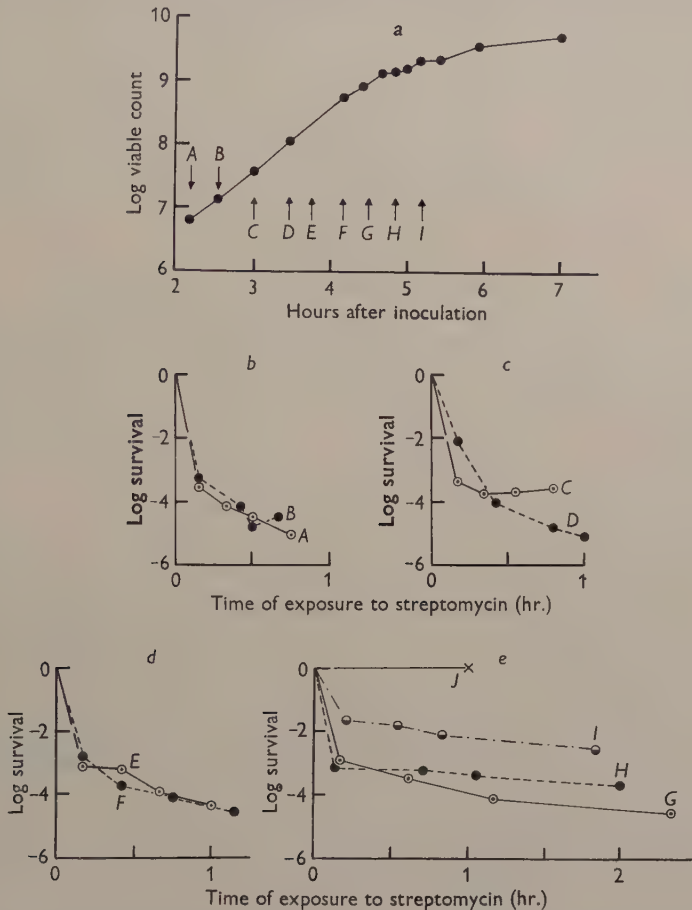


Fig. 5. Effect of streptomycin on a culture in the second half of the growth curve. At times *A*, *B* ..., shown in Fig. 5*a*, 4 ml. of culture growing with aeration at 37° were transferred to 0.44 ml. streptomycin solution at 37°, aeration being continued. The final concentration of streptomycin was 200 µg./ml. Counts were made at intervals on nutrient agar yielding the curves *A*, *B* ..., shown in Figs. 5*b-e*. Curve *J* was determined on an overnight culture.

the colony count was $10^{8.5}$, survival after chilling was 0.1, while after exposure to streptomycin for 1 hr. it was 0.0001. In another experiment, samples of the same culture were either chilled or treated with streptomycin, and again resistance to chilling was virtually complete at times when the culture was still highly susceptible to streptomycin (Fig. 6).

Experiments with Escherichia coli infected with phage T2

Doermann (1952) and Anderson & Doermann (1952) showed by disruption of phage-infected bacteria that intracellular mature phage first appeared about half-way through the latent period. These authors used either 'lysis from without', produced by the addition of a high multiplicity of phage T6 acting in the presence of 0.01 M-potassium cyanide (Doermann, 1952), or ultrasonic

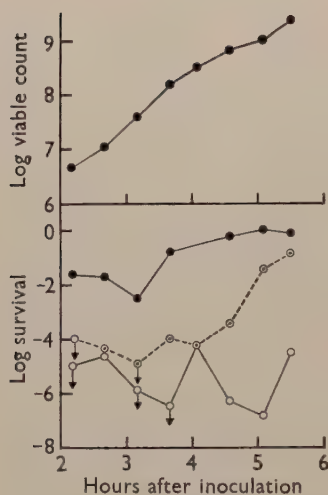


Fig. 6

Fig. 6. Survival (a) 4 hr. after sudden chilling by dilution 1/100 in distilled water at 4°, counts being made on nutrient agar with (○---○) and without (●—●) 0.25% sodium deoxycholate; and (b) 1 hr. after mixture with streptomycin solution at 37° at a final concentration of 200 µg./ml. (○—○).

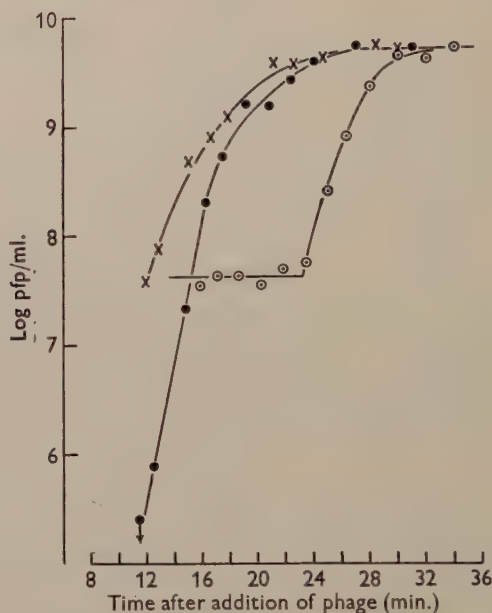


Fig. 7

Fig. 7. Effect of sudden chilling on *Escherichia coli*, strain B, infected with phage T2. An overnight culture was diluted 1/2000 in fresh broth at 37°, phage T2 being added after 3.5 hr. incubation. Antiphage serum was added 5 min. later. At 10 min., when >99% unabsorbed phage was neutralized, the culture was diluted sufficiently to prevent inactivation of liberated phage by either serum or reabsorption. The multiplicity of infection was 1.5. Samples were chilled by dilution 1/100 in distilled water at 4° and were counted 4 hr. later (●—●). Other samples were diluted 1/10 in 0.01 M-potassium cyanide in broth at 37° and were counted after 40–60 min. (×—×). The curve (○—○) is a control one-step growth curve on the same culture.

disintegration (Anderson & Doermann, 1952). After mature intracellular phage has appeared, 0.01 M-potassium cyanide alone will promptly induce lysis and gives results identical with those of the first method (Doermann, 1952).

Figure 7 shows the amount of phage released during the latent period either by 0.01 M-cyanide or by sudden chilling in distilled water at 4°. Chilling

is evidently as efficient as cyanide in extracting phage from the infected bacteria, for the counts by either method are the same at the end of the latent period (24 min. onwards) and are in turn the same as the counts obtained after spontaneous lysis. Before 24 min., the curve for chilled samples resembles that obtained by ultrasonic disintegration since at any given time, the counts are always lower than those obtained by treatment with cyanide (presumably because cyanide does not stop phage maturation instantaneously) and since less than 1 % of infected bacteria survive chilling, whereas *c.* 50 % are known to survive cyanide. Other experiments showed that sudden chilling led to maximum phage release within the succeeding 2 min. so that chilling must have acted by causing almost immediate lysis and not by arresting phage growth, followed by lysis at the normal time. Chilling in 0.3 M-sucrose at 4° gave the same counts as chilling in distilled water.

DISCUSSION

These experiments show that killing of an exponential phase culture is not due to sudden cooling in itself (since survival was complete in 0.3 M-sucrose), but evidently requires a suitable diluent as well as a sudden fall in temperature. The second condition suggests that survival after slow cooling in a potentially lethal diluent follows adaptation to the new medium by a mechanism having a negligible action at 4°. The first condition suggests that this mechanism prevents entry of water into the cell since survival was complete in sucrose, known to have this action since it can prevent plasmolysis and lysis of protoplasts of *Escherichia coli* (Lederberg, 1956). However, bacterial death following chilling was not accompanied by lysis, for chilled and unchilled samples from the same culture did not show any visible differences. The effect of sudden chilling on phage-infected bacteria is presumed to differ from its effect on normal bacteria since it was not modified by chilling in 0.3 M-sucrose. Maaløe (1950) reported that when *Escherichia coli*, strain B/1, was infected with phage T4r at 36° in broth and subsequently was suddenly cooled by dilution in broth at 19° during the last third of the latent period, some infected bacteria lysed. However, his results differ from those given above in that phage-infected bacteria were not destroyed by this degree of cooling in the first two-thirds of the latent period.

After sudden chilling of a broth culture in Ringer's solution, the survival of stationary phase organisms on nutrient agar containing 0.25 % sodium deoxycholate was *c.* 0.5, while that of exponential phase organisms was often 10^{-5} – 10^{-6} . Unfortunately, this observation could not be applied to the isolation of nutritionally exacting organisms from a predominantly prototrophic population growing in glucose+salts medium because the latter was only slightly susceptible.

It will be seen from Fig. 1 that a culture is uniformly susceptible to chilling for only a small part of the exponential phase of growth. At other times, susceptibility is either increasing or decreasing. Such heterogeneity in resistance could be accounted for by postulating pre-existent heterogeneity in

the population as would occur, for example, if the organisms differed in their requirement for some constituent of the medium so that as the count increased and the concentration of this nutrient fell, the growth rate of a fraction of the organisms fell to such an extent that it became resistant to chilling. The simplest form of this hypothesis, which supposes the survivors to comprise a non-multiplying fraction of the population, is excluded by the differing survival after sudden chilling and after exposure to streptomycin (Figs. 1, 5 and 6). It might be argued that at the moment of exposure to streptomycin, the size of the phenotypically streptomycin-resistant fraction was the same as that resistant to chilling but that it rapidly became phenotypically sensitive following death of the initially sensitive fraction. This seems improbable if depletion of a growth factor led to phenotypic resistance but is more plausible if this arose from oxygen lack. However, this is also unlikely for Paine & Clark (1953) have shown that after addition of streptomycin to susceptible cultures, oxygen consumption continues virtually unchanged for at least 1 hr. although more than 95 % cells may be incapable of colony formation.

The killing curves after either chilling or streptomycin treatment usually appeared to be smoothly concave so that the distribution of resistance to both agents could be continuous in the population. A completely resistant fraction was observed in one experiment (Fig. 5c, curve C) and it is supposed that this subculture was unfavourable to growth so that part of the population rapidly became phenotypically resistant. Differences between subcultures are presumably responsible for the gross differences in survival shown by the various subcultures treated with streptomycin in the second experiment (Fig. 6).

Several other bactericidal agents are known to act only on actively dividing cultures and, as seen above, a considerable fraction of the bacterial population may survive by virtue of its phenotype although it is clearly potentially (i.e. genotypically) sensitive. The survivors will then appear fully sensitive to the agent when retested by any method entailing multiplication (e.g. the usual antibiotic sensitivity tests). A well-known example of this phenomenon is provided by the action of penicillin on *Staphylococcus aureus* (Bigger, 1944); while Mitchison & Selkon (personal communication) pointed out that similar results are obtained with this species and streptomycin. Garrod (1948, fig. 6), also using streptomycin and *S. aureus*, reported a curve resembling curve C in Fig. 5 and found the members of the surviving fraction to be no more resistant than the original population in subsequent sensitivity tests. Survival curves showing resistant fractions of varying sizes are also obtained after exposure of cultures of *Escherichia coli* to ultraviolet light (Dr T. Alper, personal communication). In this system, as in the experiments with chilling, the behaviour of the culture can be forecast fairly well from the initial bacterial count and the time of incubation. On the other hand, little help is given by the observed rate of multiplication for (to take the extreme case where the resistant fraction was non-multiplying) the resistant fraction of the total population could clearly change from 10^{-2} to 10^{-6} , for example, without producing a detectable change in the rate of growth of the culture as a whole.

I should like to thank Dr R. C. Valentine for examining samples of chilled cells by electron microscopy, and I.C.I. Ltd. and Oxirane Ltd. for gifts of antifoam and of propylene glycol, respectively.

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Some Aspects of Soil Fungistasis

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SUMMARY: A series of fungi with varying degrees of sensitivity to soil fungistasis was selected as the result of agar disk and buried-slide tests with 17 fungi and one soil. Six out of 7 different soils exhibited a spectrum of inhibition of the test series of fungi similar to that of the first soil tested. The exception was a very acid raw humus soil which only inhibited the acid-sensitive fungus *Acrostalagmus cinnabarinus*. Fertilizer treatments of two series of plots at Rothamsted were found to affect soil fungistasis only through their influence on soil pH. Inhibitory effect decreased with increasing soil acidity, being absent from the most acid plot tested. From the results of experiments in which three fungi were pre-incubated before exposure to the influence of soil, it is suggested that spores are most sensitive to soil fungistasis at an early stage in the process of germination.

Reference was made in a previous paper (Jackson, 1958) to the numerous reports which suggest that factors which inhibit the germination of fungal spores and, to a lesser extent, the vegetative growth of fungi, are of widespread occurrence in the soil. Little is yet known of the properties and nature of the factors concerned. Dobbs & Hinson (1953) demonstrated that a considerable range of different soils all produced fungistatic effects, but that sub-soils were either less inhibitory than surface soils or without any effect. A strain of *Bacillus macerans* isolated from partially sterilized soil was shown by Park (1956) to possess the ability to digest fungal structures, to inhibit spore germination and to cause the production of distortions and sometimes chlamydospores by *Fusarium roseum*. In autoclaved soil the isolate produced toxic effects believed to be similar to those caused by fresh soil as observed by Neilson-Jones (1941) and Dobbs & Hinson (1953). The work described in the present paper was conducted to elucidate some features of soil fungistasis and particularly to determine whether or not the inhibitory effect exhibited by quite different soils is due to the presence of similar or the same inhibitory substances.

METHODS

The agar disk technique. In this technique, which has been described previously (Jackson, 1958), disks of agar 7.5 mm. in diameter by 1.5 mm. in thickness are placed on filter-paper in contact with moist unsterile soil and surface inoculated with fungal spores. After a period of incubation, the disks are examined and the % of germinated spores in 4 random fields on each of 4 replicate disks recorded. Before analysis the percentages are transformed to angular values. Control disks are incubated on moistened filter-paper in the absence of soil. As a standard procedure in the experiments described here,

disks over soil and control disks were incubated for 1 hr. at 25° after placing in contact with the filter-paper but before inoculation. The disks were prepared from agar composed of Bacto-peptone, 5.0 g.; Bacto-agar, 20.0 g.; distilled water, 1000 ml. The presence of peptone in the agar enhanced the germination of spores on control disks without interfering with the expression of fungistasis on disks over soil.

The buried slide technique. The technique used was based on that devised by Chinn (1953). Microscope slides, coated with a film of 2.0 % (w/v) agar + 0.5 % (w/v) peptone in which are suspended spores of the fungus being examined are buried in Petri dishes in 50 g. fresh soil, brought to c. 60 % of water-holding capacity with distilled water. After incubation for 42 hr. at 25° the slides are removed, allowed to dry, and stained with acetic-aniline blue (Jones & Mollison, 1948) before mounting in Euparal. One hundred spores in random microscope fields are counted and the number which have germinated recorded. Control slides also coated with spore suspensions in agar are incubated in Petri dishes over moist filter-paper and examined after 42 hr.

Soils. (1) Rothamsted I; Broadbalk field, Plot 2 (14 tons farmyard manure/acre/year), clay loam with flints overlying chalk, pH 7.2. (2) Rothamsted II; fallow plot West of greenhouses, clay loam with flints overlying chalk, pH 6.0. (3) Sewell, Bedfordshire; chalk soil containing a high percentage of raw chalk and carrying a typical mixed calcicole flora; pH 7.3. (4) Barton-in-the-clay, Bedfordshire; clay containing some chalk from a weedy pasture consisting of *Holcus*, *Agropyron*, *Trifolium*, *Ranunculus*, etc.; pH 7.3. (5) Silsoe, Bedfordshire; sandy soil with high coarse sand fraction derived from the Lower Greensand and carrying a sparse cover including *Cytisus*, *Ulex* and mixed grasses; pH 4.9. (6) Rockingham, Northamptonshire; loam containing some free limestone overlying limestone from an arable field under barley; pH 6.7. (7) Charnwood Forest, Leicestershire; acid humus soil overlying granite with cover of *Pteridium* and *Aira*; pH 2.8. (8) Sedgebrook, Lincolnshire; clay soil overlying Keuper Marl from headland of arable field; pH 6.2. (9) Baston, Lincolnshire; silty loam fen soil from arable field under wheat; pH 7.3.

Fungi. The following fungi were used, all were isolated from soil the origin of which is given: *Mucor* sp. Rothamsted; *Absidia* sp., Rothamsted; *Zygorhynchus* sp., Barnet, Hertfordshire; *Syncephalastrum* sp., Rothamsted; *Mortierella* sp., Rothamsted; *Penicillium citrinum*, Thom, Ibadan, Nigeria; *Penicillium* sp. 1, Rothamsted; *Penicillium* sp. 2, Rothamsted; *Paecilomyces marquandii* (Masse) Hughes, Woburn, Bedfordshire; *Gliocladium roseum* (Link) Bain, Woburn; *Acrostalagmus cinnabarinus* Corda, Rothamsted; *Cephalosporium* sp., Rothamsted; *Fusarium* sp. 1, Rothamsted; *Fusarium culmorum* (W. G. Sm.) Sacc. Rothamsted; *Oidiodendron* sp., Barnet; *Cladosporium herbarum* Link, Barnet; *Gliomastix convoluta* (Harz) Mason, Barnet; *Myrothecium* sp., Woburn.

RESULTS

The fungistatic spectrum of Rothamsted soil

When tested against a range of different micro-organisms, antibiotics usually produce a characteristic pattern or spectrum of inhibition as the result of differences in the sensitivities of the micro-organisms used. Since there were indications that fungi exhibit some degree of differential sensitivity towards soil fungistasis, it was considered of interest to determine the inhibitory spectrum of one soil against a range of fungi and then to compare this spectrum with those produced by other soils against fungi selected from the range.

The effect of Rothamsted I soil on the spore germination of 17 fungi was determined by the agar disk method and the buried-slide technique. On all the control slides which were incubated for 42 hr. in moist chambers the growth of fungal mycelium was too thick to allow an assessment of % spore germination to be made, but inoculated disks placed on moist filter-paper in the absence of soil provided satisfactory controls for both tests. The % germination of spores of the fungi tested on agar disks over moist filter-paper (control), agar disks over soil and on slides buried in soil are shown in histogram form in Fig. 1. The fungi have been arranged as far as possible in decreasing order of sensitivity to Rothamsted I soil in the agar disk test. It can be seen that the fungi studied varied in their reaction to soil fungistasis; for example, the spores of *Oidiodendron* sp. and *Syncephalastrum* sp. were completely prevented from germinating in the presence of soil in both tests, whereas those of *Fusarium culmorum* and *Gliocladium roseum* were not visibly affected by soil on agar disks, but were inhibited on buried slides. Buried slides were found to provide a much more sensitive test for soil fungistasis than agar disks, as might be expected from the greater proximity to the soil of spores on buried slides as compared with agar disks over soil. In the experiment for which results are given, germination of the spores of only 7 of the test fungi was observed on buried slides, while spores of 13 out of the total of 17 fungi tested showed some germination on agar disks. With one exception (*Zygorhynchus* sp.), the fungi which showed some germination on buried slides were not significantly inhibited on agar disks over soil. Although 87 out of 100 conidia of *Fusarium* sp. 1 germinated on slides buried in soil, in every case the short germ tubes produced terminated in typical thick-walled aseptate or uniseptate chlamydospores. This behaviour was never observed on agar disks over soil nor in the controls. *F. culmorum* behaved in a different manner, germination on slides in contact with soil being reduced to 2 % with no indication of chlamydospore production. The phenomenon of chlamydospore production by certain isolates of *Fusarium* in the presence of soil is being further studied.

Comparison of the fungistatic spectra of different soils

In order to compare the fungistatic spectra of a number of different soils the following fungi were chosen from those tested against Rothamsted I soil as forming a convenient test series: *Oidiodendron* sp.; *Penicillium* sp. 2;

Acrostalagmus cinnabarinus; *Cladosporium herbarum*; *Gliomastix convoluta*; *Paecilomyces marquandii*; *Fusarium culmorum* and, in addition, *Penicillium citrinum*, which had previously been used as a standard test organism (Jackson, 1958). The sensitivity of these fungi to 7 different soils was measured by the agar-disk method; the tests were all carried out on the same day and

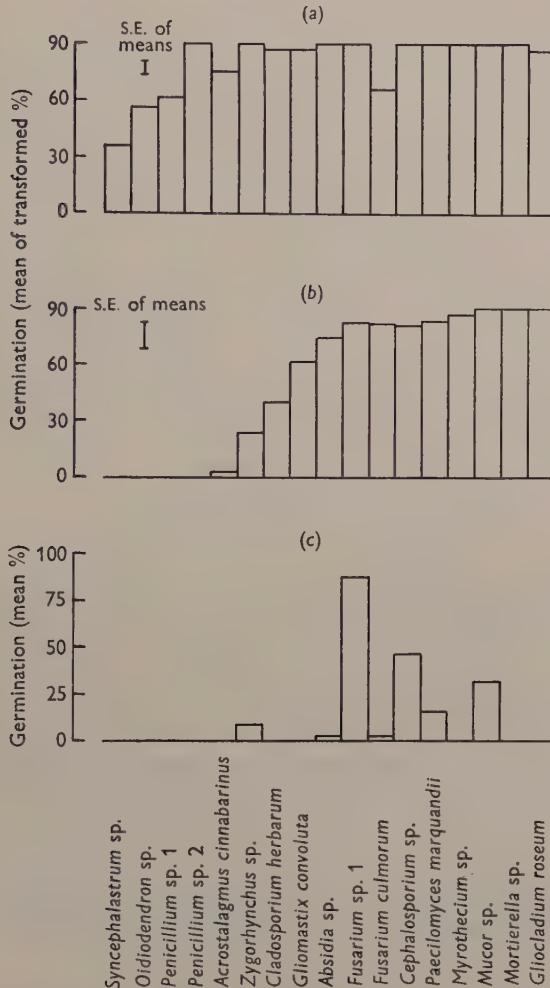


Fig. 1. Spore germination of 17 fungi: (a) on agar disks in the absence of soil; (b) on agar disks over soil; (c) on slides buried in soil. (Standard errors of means for (a) and (b) at $P=0.05$.)

within a week of collecting the samples. Before testing, the moisture content of each sample was brought up to *c.* 60% of water holding capacity with distilled water. Inspection of the results of this experiment, which are presented as a histogram in Fig. 2, reveals that, with the exception of soil no. 5, all the soils have a very similar spectrum of inhibition with respect to the 8 test fungi.

The soil which did not show a spectrum similar to that of the others was the very acid (pH 2.8) raw humus soil from Charnwood Forest; over this soil only one fungus, *Acrostalagmus cinnabarinus*, was significantly inhibited.

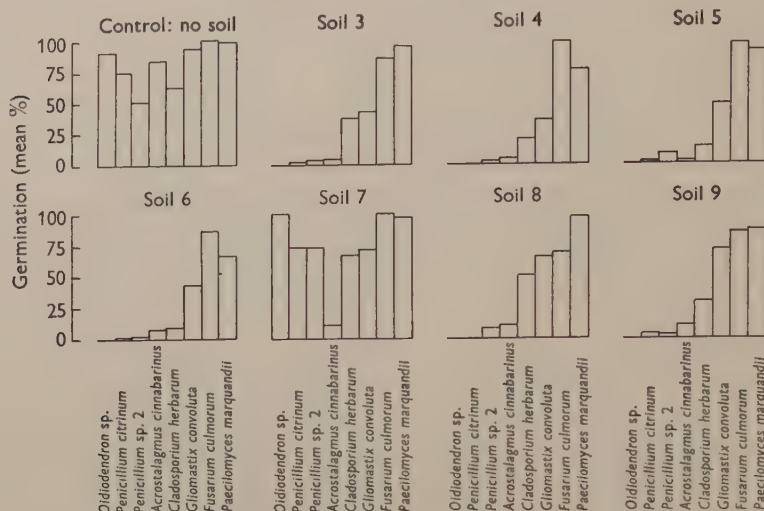


Fig. 2. Spore germination of the test series of fungi on agar disks in the absence of soil, and over 7 different soils.

Sensitivity of the test fungi to pH value

In view of the possible direct effect of soil pH value on the germination of the test fungi, tests were made using the agar-disk method to determine the effect on the germination of the test fungi of a range of McIlvaine's citric acid+sodium phosphate buffer solutions (Britton, 1955), pH 3.0-8.0. The filter-papers on which the agar disks were placed were moistened with the appropriate buffer solutions. The results of the tests (Fig. 3) show considerable differences in the effect of pH value on the germination of the test fungi. It was found, as might be expected, that the pH value of a soil had much less effect on the germination of fungal spores in the agar disk test than a buffer solution of similar pH. In the tests on different soils, the germination of only one fungus, *Acrostalagmus cinnabarinus*, was limited by the unfavourable pH of the acid raw humus soil (no. 7).

Fungistasis in Park Grass and Broadbalk plots

The classical fields at Rothamsted, the crop and fertilizer histories of which have been recorded for the past hundred years, afford a unique opportunity of studying the effects of different treatments over a long period on the fungistatic activity of soils which were originally similar. The Park Grass permanent grass plots are of particular interest since the fertilizer treatments applied have resulted in large pH differences between the plots with associated changes in the vegetation and nature of the soil. Composite soil samples were taken from

the upper 10 cm. of selected Park Grass plots and tested for fungistasis by the agar-disk method using *Penicillium citrinum* as test organism. Details of the plots sampled and the results obtained are given in Table 1. A striking feature of the results is the correlation between soil pH and inhibitory activity, strong

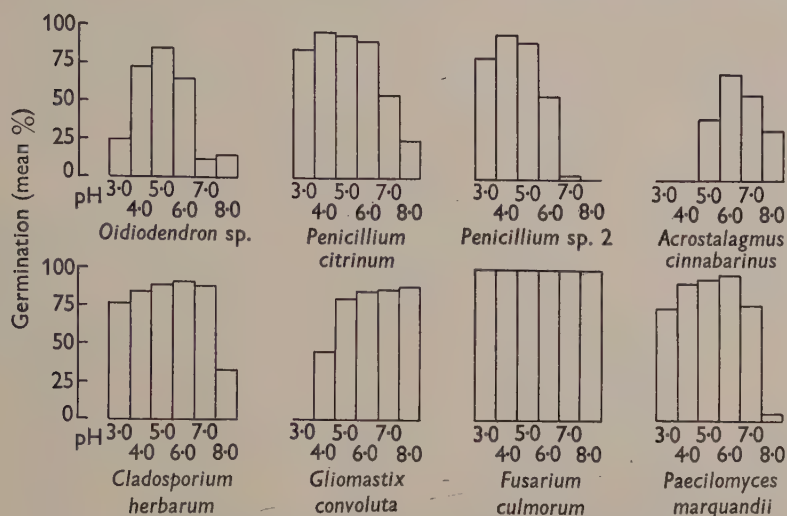


Fig. 3. Spore germination of the test series of fungi on agar disks over McIlvaine's buffer solutions.

Table 1. *The germination of Penicillium citrinum conidia on agar disks over soil from Park Grass plots*

Plot no.	Treatment	pH value	Mean % germination	Mean of transformed % germination*
3	No manure, lime	6.9	0	0
7	P, K, Na, Mg	4.5	55.7	48.4
7a	P, K, Na, Mg, lime	6.4	6.4	14.4
11 ₁	N, P, K, Na, Mg	3.0	82.7	65.6
11 ₁ a	N, P, K, Na, Mg, lime	4.3	57.2	49.3
12	No manure	5.2	10.7	18.8
13	Dung, fish guano	4.1	61.2	51.6
13a	Dung, fish guano, lime	6.4	11.5	19.5
7	As 7 above, autoclaved†	5.0	100.0	90.0
3	As 3 above, autoclaved†	6.9	100.0	90.0
Filter-paper control			88.7	70.2

N	Ammonium sulphate 6 cwt.	} per acre per year
P	Superphosphate 3½ cwt.	
K	Potassium sulphate 4½ cwt.	
Na	Sodium sulphate 1 cwt.	
Mg	Magnesium sulphate 1 cwt.	
Lime 18 cwt. per acre every 4 years		
Dung	14 tons	} per acre every four years
Fish guano	6 cwt.	

* Significant difference between means for $P=0.05$ is 5.8.

† 15 lb./sq.in. for 30 min.

inhibitory effects being associated with a high pH and weak effects with a low pH value. In each of the pairs of plots receiving lime and no lime, the limed soils produced the greater inhibition. With the most acid plot (plot 11₁) germination of *P. citrinum* conidia did not differ significantly from that of the filter-paper control. That the large differences in germination obtained with soils of different pH values was not due directly to the effect of pH value on the spores was confirmed in the tests with autoclaved soil from plots 7 and 3; 100 % germination occurred with both of these soils with pH values (after autoclaving) of 5.0 and 6.9, respectively. In this experiment pH differences between the plots obscured any effects which manurial treatments might have exerted on fungistasis.

Table 2. *The germination of Penicillium citrinum conidia on agar disks over soil from Broadbalk plots*

Plot no.	Treatment	pH value	Mean % germination	Mean of transformed % germination*
2	Dung	7.2	4.0	7.1
3	No manure	7.6	15.2	21.5
5	P, K, Na, Mg	7.5	2.3	8.2
8†	N3, P, K, Na, Mg	5.7	64.6	53.4
10†	N2	7.3	18.3	24.9
11†	N2, P	7.4	19.4	25.1
17†	N2; P, K, Na, Mg‡	7.6	3.8	11.1
Filter-paper control		—	98.6	82.2
	Dung 14 tons			
	N2 Ammonium sulphate 4 cwt.			
	N3 Ammonium sulphate 6 cwt.			
	P Superphosphate 3½ cwt.			
	K Potassium sulphate 2 cwt.			
	Na Sodium sulphate 2 cwt.			
	Mg Magnesium sulphate 1 cwt.			

} per acre per year

* Significant difference between means for $P=0.05$ is 35.3.

† Limed since 1954 to correct acidity.

‡ N2 and P, K, Na, Mg in alternate years.

On Broadbalk permanent wheat field, differences in pH values between plots due to different fertilizer treatments have been decreased since 1954 by liming the plots receiving ammonium sulphate. With the exception of plot 8 (pH 5.7), all the plots sampled had pH values between 7.2 and 7.6, although receiving different fertilizer treatments. The results of the agar-disk tests are shown in Table 2. A rather high standard deviation in the results indicates the uneven distribution of the inhibitory factor in the soil of the plots tested. Least inhibition occurred over the most acid soil, receiving the heaviest applications of ammonium sulphate, but no significant differences between the germination of *Penicillium citrinum* conidia on disks over the soils from the other plots was detected despite the differences in treatment.

*The effect of delayed exposure to soil upon spore germination
and subsequent development of fungi*

After the agar disks are placed in contact with the filter-paper in the agar-disk test, it must be assumed that a delay occurs before a sufficient quantity of the inhibitory factor diffuses into the disks to prevent germination. This assumption is supported by the observation that a delay in inoculating the disks after they have been placed in position over the soil decreases the

Table 3. *The effect of delaying the exposure to soil fungistasis of
germinating *Penicillium citrinum* conidia on agar disks*

Duration of incubation over filter-paper (hr.)	Condition on transferring to soil	Duration of incubation over soil (hr.)	Condition at end of incubation	Final % germination (mean)
0	—	24	Unswollen, ungerminated conidia and germinated conidia with well-developed normal germ tubes	12.1
4	No germination or swelling	20	Well-developed germ tubes with penicillus formation starting	74.7
8	Some conidia swollen and c. 10 % germinated	16	Well-developed branching hyphae with many penicilli-bearing conidia	*
16	c. 90 % germination and well-developed germ tubes	8	Well-developed branching hyphae with many penicilli-bearing conidia	*
24	—	0	Thick matted mycelial growth with frequent conidial heads but less abundant than above	*

* High percentage germination but hyphal development made a count impossible.

number of spores which germinate. This effect may also be examined, as in the following experiment, by allowing the spores of the test fungus to begin germination before subjecting them to the influence of the soil. Sixteen disks of peptone agar were placed in a Petri dish on filter-paper moistened with distilled water and inoculated with a suspension of *Penicillium citrinum* conidia. After 4 hr. incubation, 4 disks were examined and subsequently placed on filter-paper squares in contact with moistened Rothamsted I soil. After another 4 hr., a further set of 4 disks was similarly treated. As controls, one set of 4 disks remained on the filter-paper and another set was kept over soil for the duration of the experiment. Twenty-four hr. from the beginning of the experiment all the disks were removed and examined. The results, which are summarized in Table 3, show that a large increase in germination as compared with the soil control resulted from incubation for 4 hr. before placing the disks over soil, even though no visible changes had occurred in the spores

by the end of the 4 hr. period. After 8 hr. of pre-incubation followed by 16 hr. over soil, vegetative development had taken place to an extent comparable to the control without soil; however, conidiophore and conidium production was more abundant on the disks transferred to soil than on those remaining on filter-paper. The fungus behaved similarly on disks pre-incubated for 16 hr. before being transferred to soil for a further 8 hr. of incubation.

Further experiments on the effect of pre-incubating spores before subjecting them to the influence of soil fungistasis were made by the buried-slide technique with the two fungi *Gliocladium roseum* and *Paecilomyces marquandii*.

Table 4. *The effect of delaying the exposure of germinating Gliocladium roseum conidia to soil fungistasis*

Duration of incubation in moist chamber (hr.)	Condition on transferring to soil	Duration of incubation in soil (hr.)	Condition at end of incubation	Final % germination
0	—	48	No germination or swelling	0
3	No germination or swelling	45	No germination or swelling	0
6	No germination or swelling	18	Germinated conidia unevenly distributed	c. 4
22	c. 100 % germination, long germ tubes	26	Well-developed interwoven hyphae	c. 100
24	—	0	Well-developed germ tubes	c. 100
48	—	0	Well-developed interwoven hyphae	c. 100

Germination of the conidia of these fungi is not normally inhibited to a significant extent on agar disks over soil but is inhibited on buried slides. Rothamsted II soil was used in these buried slide experiments. The treatments and results of the experiments with *G. roseum* and *P. marquandii* are summarized in Tables 4 and 5. Both fungi responded to the treatments in a very similar manner. With up to 48 hr. of incubation in soil, or with 3 hr. pre-incubation followed by 45 hr. in soil, no visible changes occurred in the conidia of either fungus. With pre-incubation for 6 hr. before 18 hr. in soil, a small % of the conidia of both fungi had germinated although they were apparently unchanged when transferred to soil. The uneven distribution of germinated conidia on the slides following this treatment was more marked with *P. marquandii* than with *G. roseum*, but was evident with both fungi. After pre-incubation for 22 hr. both fungi had germinated well, but with *P. marquandii* differences were apparent in the final growth of the fungus between those slides which remained in moist chambers for the remainder of the 48 hr. period and those which were transferred to soil. Vegetative development of *P. marquandii* after burial in soil was considerably less than on slides which remained in the moist chamber, but phialides were more frequent and better developed.

Table 5. *The effect of delaying the exposure of germinating Paecilomyces marquandii conidia to soil fungistasis*

Duration of incubation in moist chamber (hr.)	Condition on transferring to soil	Duration of incubation in soil (hr.)	Condition at end of incubation	Final % germination
0	—	48	No germination or swelling	0
3	No germination or swelling	45	No germination or swelling	0
6	No germination or swelling	18	Germinated conidia concentrated in certain areas, short germ tubes	c. 3-20
22	c. 90 % germination well-developed germ tubes	26	Well-developed hyphae with frequent single or grouped phialides and conidial chains	c. 100
24	—	0	Well-developed germ tubes	c. 90
48	—	0	Well-developed interwoven hyphae, a few phialides present	

DISCUSSION

The comparison made when testing the response of a range of fungi to Rothamsted I soil between the behaviour of fungal spores on buried agar-coated slides and agar disks on filter-paper in contact with soil emphasizes that the latter method provides the more sensitive test for fungistasis. However, when the relative sensitivities of a number of fungi are being determined, or the inhibitory spectra of different soils compared, the smaller sensitivity of the agar disk method has certain advantages. On buried slides in Rothamsted I soil only 7 out of 17 fungi tested showed any germination, and of these, 3 gave < 10 % germination and one of the fusaria produced chlamydospores. On agar disks, on the other hand, 13 of the fungi tested germinated and it was possible to select from these a suitable test series.

The results of the tests on different soils with test series of fungi, in which similar spectra of inhibition were obtained from 6 of 7 soils, strongly suggest that identical or very similar factors or combinations of factors are operative as inhibitors of germination. If it should prove that combinations of inhibitory substances are responsible for soil fungistasis, the similarity of spectra from different soils makes it seem likely that the mixture would be a simple one with few components. The marked specificity shown towards the test fungi suggests that the inhibitor or inhibitors are complex organic substances, probably of microbial origin. Lack of inhibition of all the test fungi, except *Acrostalagmus cinnabarinus* by the very acid raw humus soil from Charnwood Forest, is in agreement with the results from the acid Park Grass plots.

The most significant finding in the experiments with soil from Park Grass and Broadbalk plots was the decrease in fungistatic effect with increasing soil acidity. Whether this was due to lack of production of the factor in acid soils

(possibly resulting from reduction in the numbers or absence of the producing organisms) or to relative biological inactivity under acid conditions such as might be shown by a basic compound, is not clear.

Two points of interest emerge from the experiments on the pre-incubation of spores before subjecting them to soil fungistasis. In the experiment with agar disks and *Penicillium citrinum* the results obtained suggest that even before visible changes have occurred in germinating conidia in the absence of an inhibitor, a stage may be reached at which normally inhibitory concentrations of the fungistatic factor fail to prevent completion of germination. As noted above, some delay is to be expected after placing a disk on filter-paper over soil before the diffusion into the upper part of that disk of an effective concentration of factor. The condition of spores on an agar disk when transferred to soil after a period of pre-incubation may not correspond to their condition at the time when the fungistatic factor begins to be effective. When buried slides are used to test the effect of soil fungistasis, on the other hand, the equilibrium concentration of inhibitor would be reached much sooner. Germination of a small percentage of conidia of both *Gliocladium roseum* and *Paecilomyces marquandii* on slides incubated for 6 hr. in a moist chamber followed by 18 hr. in soil, confirms the results obtained with *Penicillium citrinum* on agar disks and again suggests that spores are most sensitive to fungistasis at an early stage in the process of germination. With this technique also the spores had not visibly changed at the time of transfer.

Both *Penicillium citrinum* on agar disks and *Paecilomyces marquandii* on buried slides showed some increase in reproductive activity and a decrease in vegetative activity when transferred to soil after germination had started, as compared with disks or slides incubated for the whole period in the absence of soil. Although this shift in activity should not necessarily be attributed to an effect of the fungistatic factor, the possibility cannot be ignored. Christensen & Davies (1940) found that a toxic substance produced by *Bacillus mesentericus* would suppress growth, inhibit or retard spore germination and increase conidial production in *Helminthosporium sativum*. It is probable that the effects on fungi of biologically active factors present in the soil will be complex, producing reactions dependent on the stage of growth of the fungus, the concentration of the factor and the environmental conditions.

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A Defined Medium for the Growth of the Thermophilic Actinomycete *Micromonospora vulgaris*

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SUMMARY: Biotin and methionine are essential growth factors for the aerobic thermophilic actinomycete *Micromonospora vulgaris*. Biotin can be partially replaced by Tween 80 (sorbitan mono-oleate). No aerial growth is obtained in the absence of methionine. A chemically defined medium containing mineral salts, phosphate buffer (pH 6.8), soluble starch, a mixture of 18 synthetic amino acids (including methionine) and biotin will support growth of the organism.

Throughout the studies of Erikson (1952, 1953), Erikson & Webley (1953) and Webley (1954) on the aerobic thermophilic actinomycete *Micromonospora vulgaris* it was found necessary to use in the main a complex medium (C.P.S.) for growth. Erikson (1952) did, however, show that the organism would grow in the presence of Czapek's mineral salt solution containing 1% (w/v) soluble starch, 1% (w/v) vitamin-free acid hydrolysate of casein and 0.01% (w/v) autolysed yeast extract. The present work deals with the essential growth factor requirements of the organism and the establishment of a chemically defined medium for growth.

METHODS

Organism. The experiments presented here were performed with strains H, B₄, M and F of *Micromonospora vulgaris* as used by Erikson (1953).

Preparation of spore suspensions. For this purpose 2-day growth on cellophan circles over solidified C.P.S. medium was used (Erikson & Webley, 1953). The surface growth from a plate with well-developed aerial mycelium was covered with 4 ml. sterile 0.0133M-phosphate buffer (pH 6.8) and gently detached into the buffer by rubbing with a bent sterile glass rod. The suspension obtained was transferred to a 50 ml. flask containing a few sterile glass beads (3-4 mm. diameter) and shaken gently by hand for 1 min. to detach the spores which were then separated from the aerial mycelium by filtration through sterile glass wool. The spore suspension was washed four times on the centrifuge with sterile buffer and finally suspended to give about 1×10^8 spores/ml. (haemocytometer count). This suspension was preheated for 1 min. at 100° and 0.1 ml. used for inoculation.

Growth experiments. These were carried out in 100 ml. filter flasks containing 10 ml. medium. The flasks were sterilized with cotton-wool plugs in side arm and neck. After inoculation the plug in the neck was replaced by a tight-fitting rubber stopper. In this way little evaporation took place from the shallow liquid layer during the incubation period of 8-10 days at 58-60°. To

ensure a water-saturated environment for maximum production of aerial growth (see Erikson, 1953) the side arms of the filter flasks were closed by means of short pieces of pressure tubing clipped with screw clamps. No evidence of limitation of growth by lack of oxygen was observed under these conditions. The basal mineral salt solution used was as follows (% w/v): NaCl, 0.02; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02; CaCl_2 , 0.005; K_2HPO_4 , 0.05; FeCl_3 trace; pH 6.8. Soluble starch 1 % (w/v) was included in all experiments (unless otherwise stated) and further additions were made as described later. The flasks were set up in duplicate and examined daily for surface and bottom growth. Owing to the complex morphology of *Micromonospora vulgaris* (Erikson, 1953) it was not found possible to record the results of the growth experiments by conventional methods. Also, because of the inherent variability of a thermophil of such complexity of structure, it was found that not all the flasks gave uniform growth. It was therefore necessary to repeat each growth experiment several times before coming to definite conclusions. As the strains behaved similarly in their responses the results recorded are applicable to all of them.

RESULTS

Influence of increasing pH values of the medium on the growth of Micromonospora vulgaris

It was consistently observed that when the organism was grown in the presence of the basal mineral salts, soluble starch, 2 % (w/v) vitamin-free Casamino acids (Difco) and 0.01 % (w/v) yeast extract (Difco) the medium became increasingly alkaline (rising to pH 9.0) after 8–10 days. A similar effect was obtained by Baker, Sobotka & Hunter (1953) during the growth of thermophilic bacteria. With *Micromonospora vulgaris* this increasing alkalization of the medium brought about autolysis, particularly of the aerial growth. Attempts to overcome this by the use of the organic buffer recommended by Baker *et al.* (1953) were unsuccessful. Experiments were next carried out in which varying concentrations of Sørensen's phosphate mixture (pH 6.8; Clark, 1928) were added to the above medium. It was found that development of aerial growth took place in the presence of the buffer at a final concentration of 0.02M (0.717 % (w/v) $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ + 0.272 % (w/v) KH_2PO_4). Under these conditions the aerial mycelium did not rapidly autolyse and therefore in all subsequent growth experiments this buffer concentration was included.

Replacement of the vitamin-free Casamino acids by a mixture of synthetic amino acids

The first experiments in this part of the work were performed with a mixture of the 18 amino acids in hydrolysed casein according to the proportion of each present (Block, 1945) so as to give a mixture approximately equal to 2 % (w/v) vitamin-free Casamino acids. The following were the amounts (g./100 ml. culture medium): L-arginine 0.077, L-histidine 0.046, L-lysine 0.146, L-tyrosine 0.127, L-tryptophan 0.026, L-phenylalanine 0.095, L-cystine 0.0056,

DL-methionine 0.064, DL-serine 0.138, DL-threonine 0.075, L-leucine 0.181, L-isoleucine 0.12, DL-valine 0.123, L-glutamic acid 0.445, DL-aspartic acid 0.063, glycine 0.011, DL-alanine 0.056, proline 0.147. This mixture in the presence of the mineral salts and soluble starch + buffer + yeast extract gave aerial and vegetative growth of all the strains of *Micromonospora vulgaris* within 48 hr. The replacement of starch by inulin, sucrose, glucose or fructose in this medium did not improve the growth. Poor development of aerial mycelium was obtained when the starch was omitted (cf. Erikson, 1953).

Replacement of yeast extract

No growth of the organism took place in the presence of either vitamin-free Casamino acids or of the amino acid mixture used above unless (in addition to the mineral salts, buffer, and soluble starch) 0.01 % yeast extract was added. Attempts were made to replace the yeast extract by known growth factors. It was found that of those tried biotin (0.00625 $\mu\text{g./ml.}$), and to a lesser extent 1/1000 (w/v) Tween 80, could replace the yeast extract. The following well-known accessory factors singly or in combination gave negative results—vitamin B₁, riboflavin, pyridoxal, folic acid, nicotinic acid, pantothenic acid, ascorbic acid, *p*-aminobenzoic acid, inositol, β -alanine, adenosine, adenine, uracil, vitamin B₁₂, cytochrome *c* and thiocetic acid.

Tween 80 (polyoxyethylene sorbitan mono-oleate), which could partially replace biotin, could not be replaced by Tween 40 (polyoxyethylene sorbitan monopalmitate) or oleic acid. Williams, Broquist & Snell (1947) similarly found that, for growth, certain lactic acid bacteria responded to the addition of Tween 80 but not to the other two substances. These workers have shown that the growth response of lactic acid bacteria to Tween 80 is due to oleic acid provided in a non-toxic form by this compound.

It has been shown (Schaefer, Cohen & Middlebrook, 1955) that the biotin requirement of strains of *Mycobacterium tuberculosis* can be abolished when the cultures are incubated in an atmosphere containing 1–5 % (v/v) carbon dioxide. Similar attempts to replace biotin by CO₂ for the growth of *Micromonospora vulgaris* were unsuccessful.

Need for methionine for development of aerial growth

Campbell & Williams (1953) showed that certain strains of thermophilic spore-forming bacteria have absolute requirements for certain amino acids and vitamins for growth at 55°. It was therefore considered of interest to see if *Micromonospora vulgaris* also required any of the amino acids in the synthetic mixture used above as accessory factors. For this purpose growth experiments were set up, using the procedure of Campbell & Williams (1953), namely, the omission of individual amino acids from the medium containing the synthetic amino acid mixture + mineral salts + buffer + starch + biotin (0.00625 $\mu\text{g./ml.}$). It was found from these experiments that the presence of methionine was essential for the production of aerial growth; in its absence vegetative growth was obtained. Further experiments showed that methio-

nine was effective at a final concentration of 0.003 % (w/v). D-, L-, or DL-methionine were all equally effective; they could not be replaced by cysteine, cystine or thiosulphate.

Further attempts to simplify the medium

Since, with the exception of methionine, the remaining amino acids did not appear to be essential for growth, attempts were made to decrease their number in the medium. Chromatographic analysis (kindly performed by Dr R. I. Morrison of this Institute) of the culture medium after growth of *Micromonospora vulgaris* for 9 days on 2 % (w/v) vitamin-free Casamino acids + mineral salts + buffer + starch + biotin (0.00625 μ g./ml.) showed that there had been a decrease in the amounts of the following amino acids: arginine, serine, threonine, alanine, valine, isoleucine, leucine, glutamic acid, glycine, aspartic acid. Growth experiments were set up with these 10 amino acids in the usual way and methionine (0.003 % (w/v)) and biotin (0.00625 μ g./ml.) were added. After a lag period of 3–5 days, growth with production of aerial mycelium took place. It was generally inferior to that obtained with the complete amino acid mixture.

DISCUSSION

Considering its morphological complexity, the absolute growth factor requirements (biotin and methionine) of *Micromonospora vulgaris* are relatively simple when compared with certain strains of aerobic thermophilic bacteria studied by Campbell & Williams (1953). The fact that Tween 80 can partially replace biotin for growth of *M. vulgaris* indicates that oleic acid is also required by the organism. It has been suggested by Williams *et al.* (1947) that one function of biotin is to catalyse the synthesis of oleic acid. The necessity for the SH- containing amino acid, methionine, for the production of aerial growth, is of particular interest in the light of previous work (Webley, 1954), in which it was suggested that the specific inhibitory effect of oxygen on the production of aerial mycelium by *M. vulgaris* is connected with the inactivation of thiol-containing enzymes. Another feature is the poorer growth obtained in the presence of biotin and methionine when the amino acids in the defined mixture, based on vitamin-free Casamino acids, are limited to those shown to be utilized (by chromatography) during the growth of the organism. Snell (1951) pointed out that, although the explanation for this phenomenon is in most cases not known, it often lies in one of two directions, viz. an imbalance of amino acids may be present in the limited mixture, or amino acids which are not essential when omitted from a complete medium may be essential precursors in a more restricted medium. The fact that a lag period was always noted for growth in the simplified medium in this work lends support to the latter possibility operating with *M. vulgaris*.

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Hereditary Aberrancy in Growth of Some *Bacillus megaterium* Strains

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SUMMARY: Two hundred strains of *Bacillus megaterium* recently isolated from soil were tested for an antagonistic effect on a phage-resistant strain of the same species. Fifty-one strains gave a zone of inhibition on a lawn of the indicator strain. This effect was increased by a small dose of ultraviolet radiation which also caused 41 other strains to show an antagonistic effect. Sixteen strains which were completely lysed after u.v. irradiation also produced confluent lysis of the indicator strain. When grown in liquid media, these strains gave irregular growth curves owing to partial lysis during the exponential phase. Lysis was almost complete when such cultures were incubated after exposure to small doses of u.v. radiation. Lysis was always associated with accumulation of the antibacterial principal previously named 'megacin'. Irregular growth in liquid media and production of megacin were strictly correlated. However, only 2 megacinogenic strains were lysogenic and liberated phage. Populations of megacinogenic strains were examined by replica-plating for the presence of non-megacinogenic mutants. Spore suspensions of 4 megacinogenic strains contained non-megacinogenic mutants in various proportions depending on the strain and the particular sample. Non-megacinogenic mutants could not be made to lyse by exposure to u.v. radiation; did not produce any antibacterial effects; and all showed normal patterns of growth in various liquid media. It is supposed that megacinogeny and aberrant growth, as well as inducibility by u.v. radiation, of some *B. megaterium* strains are governed by a single hereditary unit (gene). It cannot be decided at present whether or not this unit is a highly defective prophage.

Some strains of *Bacillus megaterium* are capable of producing an antibacterial substance of protein character which has been named 'megacin' (Ivánovics & Alföldi, 1954, 1955). The production of this substance by growing bacteria is enhanced by irradiation with small doses of ultraviolet (u.v.) radiation which causes mass lysis and liberation of megacin. An interesting phenomenon appeared when organisms of a typical megacinogenic strain (no. 216) were grown with megacin-sensitive bacteria on the surface of solid media; plaques were formed which simulated the growth of lysogenic bacteria inoculated with an indicator strain. No phage particles could be detected in these plaques by either cultivation or electron microscopy (Ivánovics, Alföldi & Lovas, 1957).

We found recently that a considerable proportion of *Bacillus megaterium* strains showed an unusual pattern of growth in liquid medium since, after an initial period of normal growth, the turbidity of the culture decreased owing to mass lysis of the organisms. The lytic phase was in many cases only transient, as the turbidity later increased. The growth curves of these aberrant strains were variable and inconsistent, for even the same inoculum might give different curves when grown in different media. Furthermore, the results obtained in individual experiments carried out under apparently identical

conditions sometimes differed. The aim of the present investigation was to find out whether there is any relation between bacteriocinogeny and aberrant growth in *B. megaterium* strains. A study was also made to estimate the frequency of aberrant strains in soil. It will be shown below that a hereditary character may be responsible for aberrant growth.

METHODS

The methods were in general those used by Ivánovics & Alföldi (1955, 1957). Strains of *Bacillus megaterium* were isolated and identified as described by Ivánovics, Alföldi & Széll (1957) and were maintained by subculture on yeast-extract peptone (YP) agar. Strain 216 was described by Ivánovics & Alföldi (1955, 1957) and by Ivánovics, Alföldi & Lovas (1957), and it was maintained on yeast-extract casein-digest (YDC) agar. A streptomycin-resistant mutant (216S^r) was obtained on streptomycin agar.

Phages. These were the same as those described by Ivánovics, Alföldi & Széll (1957). 'Adapted' (mutant) phages, highly specific for strains 216, 605 and 638, respectively, were isolated in the usual way from either phage M₁ or phage M ii. The phages selected for adaptation had a relatively low efficiency of plating (e.o.p.) on the above strains. The e.o.p. of phage M₁ was 10^{-6} on strain 216 and 2×10^{-6} on strain 605, when the values for adapted phages was taken as 1. Phage M ii had an e.o.p. of 10^{-5} on strain 638 before adaptation. The differences between the e.o.p. values of wild type and adapted phages provided a highly reliable means for identification of individual strains.

Nutrient media. By broth is meant horse meat broth containing 1% (w/v) peptone (Richter, Budapest). Yeast extract peptone medium (YP) and yeast extract + enzymic digest of casein medium (YDC) have been described elsewhere (Ivánovics & Alföldi, 1955, 1957). Solid media contained 1.5% (w/v) agar. Agar medium for the sporulation of *Bacillus megaterium* contained 0.1% (w/v) enzymic digest of casein (Aminosol, Vitrum, Stockholm) as described by Tarr (1932).

Preparation of spore suspensions. Bacteria were grown either on Tarr's agar or on YP agar for several days at 37°. Spores formed in 95–98% of the organisms under these conditions. Spores were washed twice with saline and stored in the refrigerator.

Titration of megacin. A strain of *Bacillus megaterium*, Mut-C, which is resistant to megaterium phages, was used (Ivánovics, Alföldi & Lovas, 1957). The indicator plates used for megacin titrations were also used in a screening test for iso-antagonistic (megacinogenic) strains. In this case, a drop of a young culture of the test strain was spotted on the surface of the indicator plate and irradiated with a high pressure u.v. lamp for 6 sec. before incubation.

The source of ultraviolet light. Two sources were used: a 'Hanau' high-pressure lamp used at a distance of 25 cm., as described by Ivánovics & Alföldi (1955, 1957); and a low-pressure lamp, emitting 7 erg./sec./mm.² at 2537 Å. at 1 m. on the optical axis.

Isolation of non-megacinogenic mutants from megacinogenic strains. Stock

spore suspensions or spores harvested from cultures grown from u.v.-irradiated spores were used. In the latter case, YP plates spread with *c.* 10^7 spores were exposed to the high-pressure u.v. lamp for 160–240 sec. After incubating the plates at 37° for 4–5 days, there were *c.* 30–200 colonies, consisting mainly of spores, which were washed off and stored in the refrigerator. This material is referred to below as ‘u.v. spores’.

One-tenth ml. samples of appropriate dilutions of spore suspensions, containing 40–70 colony-formers, were spread on several meat extract agar plates, the agar content being 2.5 %. After incubation overnight at 30°, the plates were placed in an incubator at 37° and kept there until the colonies were 1.5–2 mm. in diameter. These colonies were then tested by replica-plating (Lederberg & Lederberg, 1952) to nutrient agar plates covered with an agar layer containing megacin-sensitive bacteria. Immediately after transfer of the test colonies, the indicator plates were exposed to the ‘Hanau’ lamp for 6 sec. After further incubation, megacinogenic colonies produced either clear plaques or colonies surrounded by a growth-free zone. Colonies which did not show an antibacterial effect were re-streaked from the test plate and retested several times using young cultures in YP medium. Only isolates which did not show any antagonistic effect were accepted as non-megacinogenic.

Identification of non-megacinogenic isolates. *Bacillus megaterium* strain 216 was identified by the serological specificities of its cell wall, using rabbit anti-serum in an agglutination test (Ivánovics, 1955). Resistance to streptomycin was checked by streaking on agar containing 20 µg. streptomycin/ml. agar.

The phage sensitivity of mutants was tested by spotting decimal dilutions of the phages on 10^7 organisms of the test strain incorporated in a soft agar layer. When highly specific ‘adapted’ phages were used, dilutions of the wild-type phage were included as a control.

RESULTS

The characteristics of aberrant Bacillus megaterium strains

Screening of strains by their iso-antagonistic effects. Iso-antagonistic effects between strains of *Bacillus megaterium* (Ivánovics & Alföldi, 1955) served as markers for defining groups among strains recently isolated from soil. The criteria of an iso-antagonistic effect was that the strain should produce a zone of inhibition on the surface of an indicator plate seeded with a phage-resistant strain of *B. megaterium*. Two identical indicator plates, each spotted with a suspension of the test strain, were prepared. One was u.v. irradiated before incubation, while the second, which was not exposed to u.v. radiation, served as a control. The test strains were then classified by their colonial appearance, as follows: (1) no inhibition around the colony; (2) an inhibition zone not exceeding 2 mm. in width; (3) a zone greater than 2 mm. in width; and (4) a growth-free area of diameter 10–14 mm. at the site of inoculation. Irradiation of the plates before incubation either induced or enhanced the iso-antagonistic effect with many of the strains. Table 1 gives 200 strains, recently isolated from

soil, classified in this way. Sixteen strains gave maximum inhibition on the indicator plates and these form the main subject of the following study.

Table 1. *Classification of 200 strains of Bacillus megaterium according to their iso-antagonistic effect*

No u.v. irradiation		After u.v. irradiation	
No effect	149	No effect	108
		Zone appeared	38
		Clear plaque	3
Zone less than 2 mm.	44	Same as without u.v.	5
		Zone increased	29
		Clear plaque	10
Zone broader than 2 mm.	7	Same as without u.v.	0
		Zone increased	4
		Clear plaque	3
Total	200		200

Cultivation of strains of Bacillus megaterium in liquid media. Media were inoculated from an overnight YP or YDC agar culture and growth of the organism followed by measuring the optical density of the cultures, aeration and turbidity measurements being carried out as described by Ivánovics & Alföldi (1955, 1957). For technical reasons, the experiments were ended after 10 hr. A typical megacinogenic strain (no. 216; Ivánovics & Alföldi, 1955, 1957) was also studied. All the 17 strains which showed a marked iso-antagonistic effect in screening tests were grown several times in different liquid media during these investigations over a period of nearly two years. In one series of experiments, only 3 strains gave a normal curve, the remaining 14 being aberrant. By 'normal' growth, we mean logarithmic increase after a lag phase of 3 hr. The shapes of the aberrant curves were rather variable; curves from some typical experiments are shown in Figs. 1 and 2.

The growth curve of a given strain might vary markedly from one experiment to the next, although all experiments were carried out under the same conditions. Figure 3 shows variable results obtained with strain 605. The variability of the growth curves should not be related to any detail of the experimental technique. However, the medium generally had a marked effect on the outcome of the experiment. Thus, some strains gave very aberrant curves when grown in YP medium while the same inoculum grew normally in another medium, such as YDC medium. We are not able to give a reasonable explanation for the observed discrepancies between individual experiments so that the lysis of these strains of *Bacillus megaterium* has at present to be attributed to an 'uncontrollable' factor or factors.

Eight strains which did not show an iso-antagonistic effect even after exposure to u.v. radiation were also included for comparison. All these strains gave normal growth in all media tested. Furthermore, aberrant growth could not be produced even by a small dose of u.v. radiation. Similar results were obtained with 6 strains which showed a moderate iso-antagonistic effect

(i.e. a zone of less than 2 mm. diameter, not increased by u.v. irradiation). All the above observations support our assumption that a marked iso-antagonistic effect in the screening test was well correlated with aberrant growth in liquid medium. Also, the degree to which iso-antagonistic effects are enhanced by u.v. irradiation serves as an efficient marker for the isolation of strains exhibiting aberrant growth.

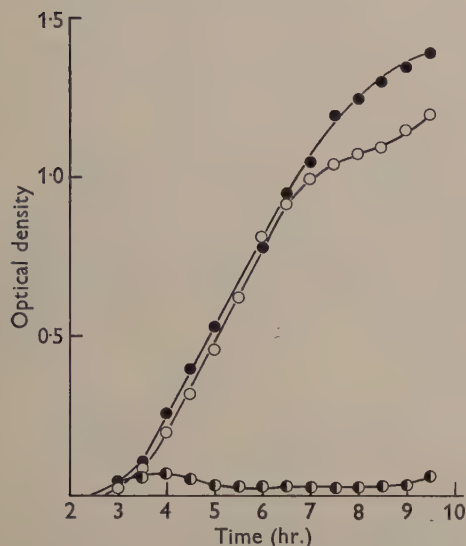


Fig. 1

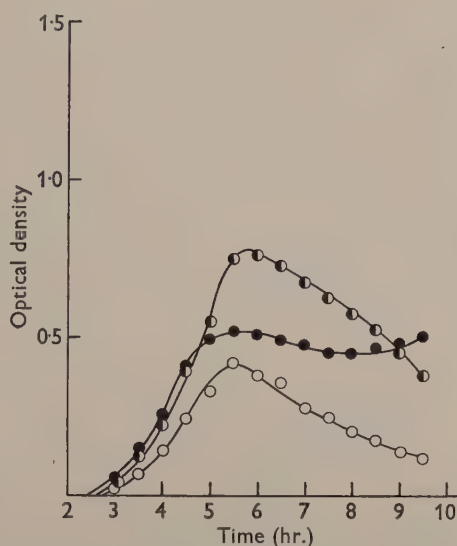


Fig. 2

Fig. 1. Growth of *Bacillus megaterium* strain 594 in various media. Media were inoculated from YDC agar. Size of inoculum: 3×10^6 colony-formers. Amount of medium: 10 ml. ●—●, meat extract; ○—○, YP; ◐—◐, YDC.

Fig. 2. Growth of *Bacillus megaterium* strain 604 in various media. ●—●, meat extract; ○—○, YP; ◐—◐, YDC.

Investigation of the aberrant strains for lysogeny. Although the indicator strain used in the screening test for iso-antagonistic effects was assumed to be entirely resistant to megaterium phages, we had also to consider whether the antagonistic effect and its enhancement by u.v. irradiation was due to a carried temperate phage. The difficulty in detecting small amounts of temperate phage in cultures of strains which may simultaneously elaborate megacin is that the latter kills the indicator strain when lysates or supernatant fluids of the cultures are assayed for infective centres. This was overcome by separating hypothetical phage from the culture by high-speed centrifugation of supernatant fluids, after sterilization by streptomycin, and subsequent assay for free phage by plating the deposit on a streptomycin-resistant indicator strain.

Each of the 16 aberrant strains was examined. Cultures in YDC medium containing 2.5×10^8 – 3.5×10^8 bacteria/ml. were first centrifuged at 3000 rev./min. for 20 min. to remove most of the bacteria. The supernatant fluids were

collected and streptomycin (20 $\mu\text{g./ml.}$ suspension) added to prevent the growth of residual bacteria. Six ml. of supernatant fluid were then centrifuged at 100,000 g for 1 hr., the supernatant fluid discarded and the pellet resuspended in YP medium. The suspension was again centrifuged at 100,000 g for 1 hr., and the pellet suspended in 6 ml. YP medium containing streptomycin. One ml. of each sample was then plated with a streptomycin-resistant phage-sensitive indicator strain (*Bacillus megaterium*, strain 337a, 337b or KM) in an agar layer containing streptomycin. Fourteen of the 16 aberrant strains did not produce any plaques; that is, *c.* 10^9 organisms of these strains did not yield even one infective phage particle. Two strains (508 and 597) proved to be lysogenic and gave small numbers of infective centres, the observed yield being 1 plaque from 50 bacteria of strain 508 or from 400 bacteria of strain 597. The efficiency of the above method was checked by treating a suspension of the megaterium phage M_1 (10^{10} particles/ml.) in the same way; 98 % of the original phage was recovered. These results show clearly that most aberrant, that is megacinogenic, strains are non-lysogenic. Two strains yielded small numbers of plaque-forming particles, however, so that lysogeny and megacinogeny can occur simultaneously in some strains.

Table 2. *The rate of growth and lysis expressed in optical density values of irradiated and control cultures of 17 strains of Bacillus megaterium in YDC medium*

Young exponentially growing cultures at an optical density (OD) of 0.2 were u.v.-irradiated. After re-incubation the extent of residual growth is expressed by the peak value OD, and the extent of lysis is expressed by the OD values measured at the end of experiment. The *antibacterial titre* is expressed by the reciprocal of highest effective dilution of supernatant fluid. Zero means no antibacterial effect undiluted material.

Strain	Irradiated cultures			Non-irradiated culture		
	Peak value	At the end of experiment	Anti-bacterial titre	Peak value	At the end of experiment	Anti-bacterial titre
	Rel. optical density values			Rel. optical density values		
216	0.56	0.04	40,000	1.10	1.10	0
507	0.35	0.04	100	1.40	1.40	0
508	0.26	0.04	10	1.20	1.20	0
519	0.56	0.13	100			
527	0.25	0.04	1	1.30	1.30	0
575	0.50	0.20	10	1.30	1.30	0
594				0.20	0.07	100
597	0.50	0.15	10	1.30	1.30	0
598	0.50	0.11	100	1.30	1.30	0
604	0.27	0.09	10	0.30	0.10	1
605	0.28	0.03	10	1.40	1.40	0
637	0.50	0.13	10	1.35	1.35	0
638	0.60	0.40	1	1.40	1.40	0
659	0.25	0.04	10	1.05	1.05	0
669	0.28	0.04	10	1.30	1.30	0
675	0.22	0.05	10	1.20	1.20	0
681	0.30	0.08	10	1.30	1.30	0
689	0.53	0.17	100	1.40	1.40	0

Lysis induced by u.v. irradiation of cultures of Bacillus megaterium. Twenty ml. portions of YDC medium were inoculated from an overnight agar culture of the test strain and aerated by gentle shaking at 35°. When the viable count was $c. 3 \times 10^7$ /ml., 10 ml. were placed in a Petri dish and irradiated with a high-pressure lamp for 60 sec. The irradiated sample, and also a control un-irradiated culture, were then re-incubated. The megacin content of each culture was titrated at the end of each experiment. Table 2 shows that all the aberrant strains tested, lysed after irradiation and re-incubation. However, the amount

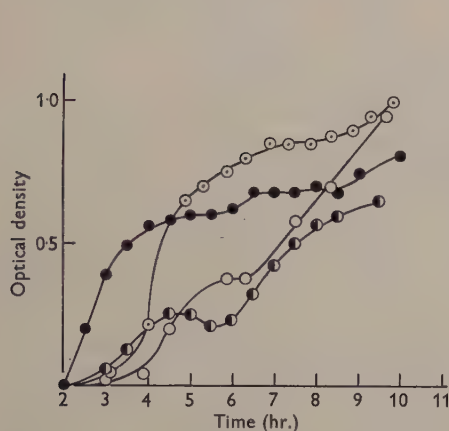


Fig. 3

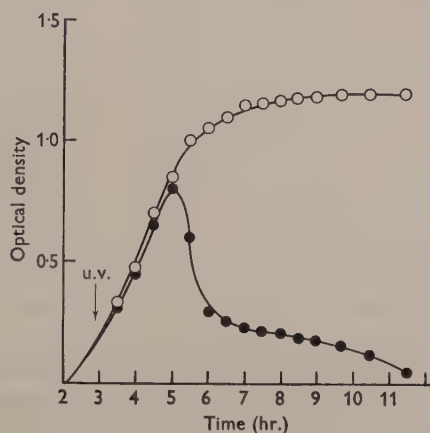


Fig. 4

Fig. 3. Growth of *Bacillus megaterium* strain 605 in horse meat extract broth in several experiments. Date of experiments: \odot , 30. v. 1956; \bullet , 5. vi. 1956; \bullet , 2. i. 1957; \circ , 16. v. 1957.

Fig. 4. Growth of *Bacillus megaterium* strain 216 and its 216 meg⁻ mutant after u.v. irradiation. Megacin titres at the end of cultivation: 1/40,000 in lysate of 216. No antibacterial effect was shown by the supernatant fluid of strain 216 meg⁻. \circ — \circ , strain 216; \bullet — \bullet , strain 216 meg⁻.

of residual growth, that is, the maximum optical density after irradiation, differed considerably with different strains. In some cases, the un-irradiated sample also showed early lysis (see strains 519 and 594 in Table 2). It must be stressed that early and apparently spontaneous lysis occurred more often when broth or YP medium was used instead of YDC medium. The megacin titres of cultures lysed by u.v. irradiation also differed enormously according to the strain. The titres appeared to be influenced also by the degree of lysis and by the maximum growth that was reached before lysis. When no detectable lysis occurred, as in the case of the un-irradiated of each culture, no megacin formation could be detected.

Several attempts were made to detect the presence of infective phage particles in lysates of irradiated cultures but only two of the strains mentioned above yielded phage, namely, 508 and 597, whose cultures contained respectively 2×10^6 and 5×10^8 plaque-forming particles/ml. A number of strains which did not show an iso-antagonistic effect in the screening test were also

included in these experiments. None of them could be made to lyse by u.v. irradiation in various liquid media. Accordingly, no antibacterial effect was shown by supernatants of their cultures.

Analysis of populations of megacinogenic strains

General considerations. All the experiments described so far used inocula prepared from confluent growth on an agar slope and not from a single colony. The inocula were thus heterogeneous in origin. It should, however, be borne in mind that even when a suspension of vegetative *Bacillus megaterium* is used, colonies forming on solid media cannot be considered to be pure clones because of chain formation by this species. Homogeneous clones can only be obtained from a well-dispersed suspension of spores.

Spore suspensions were therefore used exclusively in the study of the genetic composition of the megacinogenic strains. Any vegetative organisms present (c. 5 %) appeared to be degenerate, for their staining characteristics suggested advanced lysis. These remnants of non-sporing organisms have been disregarded below, whether or not they were capable of forming colonies. It would have been easy to get rid of these organisms by heat shock but this treatment was avoided because we feared that some spores of low heat-resistance might be killed, so leading to a change in the composition of the population. Our aim has been to isolate non-megacinogenic mutants from strains which showed iso-antagonistic effects as well as aberrant growth when grown in liquid media. It was hoped that u.v. irradiation of stock spore suspensions would promote the appearance of such mutants, on the assumption that irradiation would lead to lysis of the megacinogenic members of the population (Ivánovics, Alföldi & Lovas, 1957).

Any clone isolated from a megacinogenic strain which did not show an iso-antagonistic effect when tested on indicator plates, even after u.v. irradiation, was designated 'meg⁻', provided this character remained stable on sub-culture. For example, 216 meg⁻ describes such a clone isolated from the megacinogenic strain 216. The identity of these clones was checked by their sensitivity to 'adapted' highly specific phages; or when these were not available, by the use of a set of megaterium phages differing in host range (Ivánovics, Alföldi & Széll, 1957). Other markers used were the serological character of the cell wall (Ivánovics, 1955; Ivánovics, Alföldi & Széll, 1957) and resistance to streptomycin. A non-megacinogenic mutant was accepted as a descendant of the test strain if it shared at least one marker character with the parent strain.

Experiments with strain 216. This strain is of particular interest because it is highly megacinogenic. Table 3 gives details of experiments carried out with 216, which was extremely sensitive to streptomycin, and with streptomycin-resistant mutant of it, designated 216 Sr. Both the mutant and parent stocks were attacked to the same extent by an 'adapted' phage. In 7 experiments, 8798 separate colonies were tested by replica-plating to indicator plates sown with a megacin-sensitive strain. Altogether, 52 colonies were found which did not produce an iso-antagonistic effect after small doses of u.v. radiation. These

strains were proved to be non-megacinogenic on further investigation. Thirty-two non-megacinogenic colonies were isolated from strain 216 S^r in the same way. All the non-megacinogenic strains possessed the same phage sensitivity, antigenic structure and resistance to streptomycin as the parent strain.

Table 3. *Frequency of non-megacinogenic mutants in spore suspension of strains 216 and 216 S^r of Bacillus megaterium*

No. of experiment	Strain	Spore material	No. of colonies examined	No. of non-megacinogenic colonies
1	216	Stock material	1167	2
2	216	'u.v. spore' 28/29	1016	0
3	216	'u.v. spore' 55/61	438	11
4	216	'u.v. spore' 69	985	7
5	216 S ^r	'u.v. spore' 55/57	994	2
6	216 S ^r	'u.v. spore' 56/72	1436	15
7	216 S ^r	'u.v. spore' 56/74	2762	15

The non-megacinogenic state has proved to be stable, for no back mutants have yet been detected during a large number of subcultures. In one experiment, one 216 meg⁻ isolate was kept in the logarithmic phase of growth by serial transfer to fresh medium, samples of the culture being tested at intervals by plating in an agar layer with megacin-sensitive cells; but no megacinogenic cells were isolated from the several hundred million cells cultivated.

None of the 52 non-megacinogenic isolates could be induced to lyse by exposure of cultures in liquid medium (YDC) to u.v. radiation. Nor did the supernatant fluids of irradiated cultures show any antibacterial effect when tested on indicator plates after re-incubation, thus proving a complete lack of megacin production by these cultures. Figure 4 illustrates the outcome of such an experiment. Another observation made during cultivation of the non-megacinogenic mutants was that these invariably gave normal growth curves so that loss of megacinogeny appears to be paralleled by loss of an aberrant pattern of growth as well as by loss of inducibility by u.v. radiation.

Experiments with other megacinogenic strains. Experiments similar to those described above were carried out with 4 megacinogenic strains of different origin. Strain 597, besides being megacinogenic, liberated a temperate phage during exponential growth, and lysis after u.v. irradiation was accompanied by mass liberation of phage particles. This strain, and also strain 575, were identified by their patterns of sensitivity to a set of *Bacillus megaterium* phages. In the case of strains 605 and 638, highly specific 'adapted' phages were available for identification.

Table 4 shows that the proportion of non-megacinogenic isolates varied in different experiments. Two isolates were obtained from the lysogenic strain 597, one of which proved to have lost its lysogenicity, while the second carried the same phage as the parent strain. The lysogenic isolate was, however, no longer inducible by u.v. radiation.

A high proportion of non-megacinogenic mutants were isolated from strain

605. No differences were found when either stock or 'u.v. spore' material was used. We consider that it is unjustifiable to conclude from the difference observed between the frequency of non-megacinogenic mutants obtained in these two experiments that u.v. irradiation promotes the isolation of these mutants.

Table 4. *Frequency of non-megacinogenic mutants in spore suspension of individual megacinogenic strains of Bacillus megaterium*

Strain	Spore material	No. of colonies examined	No. of non-megacinogenic colonies
575	'u.v. spore' 56	1746	5
597	'u.v. spore' 54	1065	0
597	'u.v. spore' 56	1033	2
605	Stock material	359	35
605	'u.v. spore' 54	714	63
638	Stock material	652	0
638	'u.v. spore'	1086	22

Sensitivity of spores of the parent and the non-megacinogenic strains to u.v.-irradiation. Although wild type organisms were lysed when their exponentially growing cultures were irradiated and re-incubated, lysis did not occur when spores of these strains were irradiated and plated on agar. Spore suspensions of strains 216 and 216 meg⁻ were washed twice with saline, poured into Petri dishes to give a layer not more than 2 mm. thick, and then irradiated with a low-pressure mercury lamp while the dish was gently swirled by hand. At intervals during irradiation, appropriate dilutions were plated on YP or on meat-extract agar and incubated. There was no significant difference between the survival curves of megacinogenic and of non-megacinogenic spores, both appearing to be equally sensitive to u.v. radiation. This implies that spores are not induced to lyse by u.v. radiation even when they are plated immediately after irradiation. The shapes of the curves suggest a multi-hit mode of killing. These results are in sharp contrast with our previous observations on the inducibility of exponentially-growing cultures (Ivánovics & Alföldi, 1957).

DISCUSSION

Systematic investigations involving 200 strains of *Bacillus megaterium* recently isolated from soil provide further data concerning the occurrence of strains capable of producing an antibacterial principle identical or similar to that which has been described recently and named 'megacin' (Ivánovics & Alföldi, 1954, 1955). Nearly half these strains elaborated megacin after exposure to small doses of u.v. radiation. The release of the antibacterial principle follows partial or complete lysis of the cultures and is enhanced or induced by the small doses of u.v. radiation acting on growing organisms. Strains which were highly inducible also displayed irregularities in their growth curves when cultivated in different complete media. It is supposed

that the aberrant growth pattern of these strains is elicited by some unknown agents which exist or appear during logarithmic growth in liquid media. Megacinogeny and aberrant growth pattern are therefore different manifestations of some factor occurring in a large proportion of *Bacillus megaterium* strains.

The behaviour of these aberrant strains is in many ways reminiscent of the behaviour of lysogenic bacteria. Nevertheless only 2 of 16 aberrant strains yield infective phage particles from their cultures; the remaining 14 strains did not yield even 1 infective particle/ 10^9 bacteria. Similarly, no infective particles could be demonstrated in cultures of megacinogenic strains after mass lysis had been induced by u.v. irradiation. Lysogeny and megacinogeny may coexist in the same bacterium as was shown in the case of 2 megacinogenic strains.

We think it can safely be said that megacinogeny is not necessarily associated with lysogeny, at least, not with a lysogenic relationship which results in the liberation of infective phage particles. However, the possibility of non-infective phage production is not excluded. A thorough study of strain 216, involving electron microscopy of lysing cells (Ivánovics, Alföldi & Lovas, 1957), did not reveal any structures reminiscent of defective phage production. Such a study, however, only excludes incomplete phage production at a morphological level and does not disprove a possible synthesis of some non-organized phage material governed by a highly degraded prophage.

As non-megacinogenic mutants were isolated from megacinogenic strains, it is safe to assume that there is a factor perpetuated in megacinogenic bacteria which governs the hereditary character of megacinogeny. This gene, whether located in the chromosomal or in the cytoplasmic substance of the organism, is responsible for phenomena such as inducibility to lysis, which in turn also shows itself by an aberrant pattern of growth in liquid media, as well as by megacin production. The genomes of *Bacillus megaterium* strains differ as to megacinogeny. Therefore, our observations may be of significance in the ecology of this species.

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Induction of Bacteriolysis by Cysteamine and Its Derivatives

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SUMMARY: Cysteamine and certain closely related derivatives induced lysis in a majority of 28 strains of *Bacillus*; cultures of all other genera tested were resistant. Forty-three additional sulphur and/or nitrogen containing compounds were unable to induce lysis. Optimum conditions for cysteamine activity included exposure of metabolizing post-log. phase organisms contained in shallow shaken layers of nutrient broth to the compound for 4-8 hr. Upon the completion of lysis, no formed cellular elements were visible by microscopy except flagella. The induction by cysteamine of a lysogenic bacteriophage or of an unusually large amount of an autolytic enzyme was not demonstrated.

Among the substances which provide some degree of protection to living cells subsequently exposed to ionizing radiation is a group of chemical compounds consisting of cysteamine (β -mercaptoethylamine; MEA) and certain closely related derivatives. A preliminary report concerning the ability of the oxidized form of cysteamine (cystamine: 2,2-dithiobis-(ethylamine)) to induce lysis of organisms of various *Bacillus* spp. has been published (Weinberg, 1957). The present paper describes tests for induction of lysis with other members of the cysteamine group and with 43 related compounds. Various hypotheses concerning the mechanism of lytic induction are considered and the results of experiments designed to test some of these hypotheses are presented.

METHODS

Organisms. The test organisms (Table 1) were obtained from the collection of the Department of Bacteriology, Indiana University, except for the culture of *Micrococcus lysodeikticus* which was kindly supplied by Dr Arthur Schade, National Institutes of Health (Bethesda, Maryland, U.S.A.). Each strain was checked for purity by the usual cytological and cultural methods and was then maintained on nutrient agar (Difco) slopes. Subcultures on fresh nutrient agar slopes were made weekly.

Culture media. The majority of the experiments were performed with organisms grown in nutrient broth (Difco). In a few tests, specified in the section on results, a chemically defined medium consisting of the following ingredients, at the molarities (M) indicated, dissolved in triple-distilled water was used: 1×10^{-2} , glucose; 1×10^{-2} , NH_4NO_3 ; 9×10^{-3} , Na_2HPO_4 ; 6×10^{-3} , KH_2PO_4 ; 3×10^{-4} , MgSO_4 ; 2×10^{-5} , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; pH value, 7.0. Ten ml.

quantities of the nutrient broth or defined liquid medium were added to 50 ml. Erlenmeyer flasks and sterilized by autoclaving.

Compounds tested for ability to induce lysis. The compounds tested for the ability to induce lysis or to affect lysis-inducing compounds are listed in Table 2. The compounds were dissolved in triple-distilled water and sterilized by autoclaving or, with the heat labile substances, by filtration through sintered glass filters. Solutions of the compounds were added aseptically to flasks of inoculated culture medium at (a) the beginning of the lag phase, or (b) during the post-log. phase. When not in use, the solutions were stored at 4°; for some tests, however, fresh solutions of the cysteamine compounds were prepared daily.

Table 1. *Susceptibility of test strains of Bacillus and other genera to induction of lysis by cysteamine*

Cysteamine ($2 \times 10^{-3}M$) was added to post-log. phase cultures. Turbidity readings were made during the subsequent 24 hr. period of incubation. Of the sensitive strains, the majority of the *B. subtilis* group lysed within 4–8 hr.; the majority of the other groups required 8–24 hr. A dark pigment was produced from cysteamine by 1 sensitive and 1 resistant strain of *B. megaterium* and by 1 resistant strain of *B. cereus*.

Genus	Species	No. of strains tested	No. of resistant strains	No. of sensitive strains
<i>Bacillus</i>	<i>subtilis</i>	7	0	7
	<i>megaterium</i>	6	1	5
	<i>cereus</i>	4	1	3
	<i>macerans</i>	2	1	1
	<i>anthracis</i>	2	1	1
	<i>sphaericus</i>	1	0	1
	<i>brevis</i>	1	1	0
	<i>circulans</i>	1	1	0
	unidentified	4	3	1
<i>Sarcina</i>	<i>lutea</i>	1	1	0
<i>Staphylococcus</i>	<i>aureus</i>	1	1	0
<i>Micrococcus</i>	<i>lysodeikticus</i>	1	1	0
<i>Mycobacterium</i>	<i>avium</i>	1	1	0
<i>Escherichia</i>	<i>coli</i>	1	1	0
<i>Salmonella</i>	<i>typhimurium</i>	1	1	0

Inocula and conditions of incubation. Flasks of nutrient broth or defined liquid medium were inoculated with organisms from the agar slopes and were shaken in a New Brunswick shaker (160 strokes/min.) at 37°. After 16 hr., the cultures were diluted in fresh nutrient broth or defined medium so that 1 ml. of the diluted culture contained approximately 2×10^6 viable organisms. One-twentieth ml. portions of the diluted culture were added to the flasks to be used in the actual experiments. The experimental flasks were placed in the shaker and incubated at 37° for periods from 1 to 6 days.

Determination of bacterial multiplication and lysis. The organisms of the inocula were observed to multiply and, in some cases, subsequently to lyse by turbidimetric measurements made with the Junior Coleman spectrophotometer model 6A (at 660 m μ). To confirm multiplication and subsequent lysis

Table 2. *Compounds tested for ability to induce lysis of Bacillus subtilis strain Marburg*

Each compound was added to 16 hr. nutrient broth cultures to obtain a final concentration of 1×10^{-3} M. Turbidity readings were made at 16 hr. and at appropriate intervals up to 40 hr. of incubation.

I. Substituted ethanes

Ethyl amine	$\text{CH}_3 \cdot \text{CH}_2 \cdot \text{NH}_2$
Ethylene diamine	$\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{NH}_2$
Thio ethane	$\text{CH}_3 \cdot \text{CH}_3 \cdot \text{SH}$
Cysteamine	$\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{SH}$
Taurine	$\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{SO}_3\text{H}$
Ethanol	$\text{CH}_3 \cdot \text{CH}_2 \cdot \text{OH}$
Ethylene chlorohydrin	$\text{Cl} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$
Ethanol amine	$\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$
β -Mercaptoethanol	$\text{HS} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$
Acetic acid	$\text{CH}_3 \cdot \text{COOH}$

II. Substituted acetic acids

Thiolacetic acid	$\text{CH}_3 \cdot \text{COSH}$
Thioglycollic acid	$\text{HS} \cdot \text{CH}_2 \cdot \text{COOH}$
Sulphoacetic acid	$\text{HO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{SO}_3\text{H}$
Glycine	$\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{COOH}$

III. Substituted propionic acids

L-Alanine	$\text{CH}_3 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$
DL-Serine	$\text{HO} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$
β -Alanine	$\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$
Mercaptopropionic acid	$\text{HS} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$
L-Cysteine	$\text{HS} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$
L-Cysteic acid	$\text{HO}_3\text{S} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$
D-Penicillamine	$\text{HS} \cdot \text{C}(\text{CH}_3)_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$

IV. Mono- and di- thio ethers

Thiodiethanol	$(\text{HO} \cdot \text{CH}_2 \cdot \text{CH}_2)_2\text{S}$
Mercaptosuccinic acid	$(\text{HOOC} \cdot \text{CH}_2)_2\text{S}$
Aminoethyl sulphide	$(\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2)_2\text{S}$
Cystamine	$(\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{S})_2$
L-Cystine	$(\text{HOOC} \cdot (\text{NH}_2)\text{CH} \cdot \text{CH}_2 \cdot \text{S})_2$
L-Djenkolic acid	$(\text{HOOC} \cdot (\text{NH}_2)\text{CH} \cdot \text{CH}_2 \cdot \text{S})_2\text{CH}_2$

V. Substituted butyric acids

DL-Homocysteine	$\text{HS} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$
DL-Methionine	$\text{H}_3\text{C} \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$

VI. Isothiuronium compounds

Aminoethyl isothiuronium bromide (AET)	$(\text{Br} \cdot \text{H}_3\text{N})(\text{HN}:)\text{C} \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{HN}_2$
S, γ -Aminopropyl isothiuronium bromide (APT)	$(\text{Br} \cdot \text{H}_3\text{N})(\text{HN}:)\text{C} \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{HN}_2$

Table 2 (cont.).

VII. Pyrrolidine, thiazole, thiazoline, and thiazolidine compounds

L-Proline	$\text{CH}_2 \cdot \text{CH}_2 \cdot \text{NH} \cdot \text{CH}(\text{COOH}) \cdot \text{CH}_2$
2-Aminothiazole	$\text{S} \cdot \text{C}(\text{NH}_2) : \text{N} \cdot \text{CH} : \text{CH}$
Rhodanine	$\text{S} \cdot \text{C}(:\text{S}) \cdot \text{NH} \cdot \text{C}(:\text{O}) \cdot \text{CH}_2$
2-Methyl thiazoline	$\text{S} \cdot \text{C}(\text{CH}_3) : \text{N} \cdot \text{CH}_2 \cdot \text{CH}_2$
2-Mercapto thiazoline	$\text{S} \cdot \text{C}(\text{SH}) : \text{N} \cdot \text{CH}_2 \cdot \text{CH}_2$
Thiazolidine-4-carboxylic acid	$\text{S} \cdot \text{CH} : \text{N} \cdot \text{CH}(\text{COOH}) \cdot \text{CH}_2$
2,4-Thiazolidine dione	$\text{S} \cdot \text{C}(:\text{O}) \cdot \text{NH} \cdot \text{C}(:\text{O}) \cdot \text{CH}_2$
2-Thiazolidine thione	$\text{S} \cdot \text{C}(:\text{S}) \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CH}_2$

VIII. Miscellaneous compounds

Biotin
 1,2-Diaminocyclohexane tetraacetic acid
 Ethylene diamine tetraacetic acid
 Glutathione
 Nitrogen mustard
 Pantetheine
 Putrescine
 Thiamine
 Trypsin

as indicated by the increase and decrease in turbidity, plate counts of viable organisms and microscopic examinations were made of the cultures at appropriate intervals. The microscopic examinations included: Gram staining, cell-wall stain (Dyar, 1947), observation of wet mounts by phase microscopy, and photographs of organisms under electron microscopy.

Extraction of possible cysteamine-induced lytic principles. Organisms exposed to cysteamine added to the medium at the time of inoculation were ruptured by sonic oscillation or by ballotini in the Mickle tissue disintegrator at 6 hr., 10 hr., and 14 hr. after inoculation. Extracts of organisms not exposed to cysteamine were similarly prepared at the same times. The culture filtrates from 6, 10, and 14 hr. cysteamine-exposed and unexposed organisms were saved. Some cultures containing cysteamine were permitted to lyse and the lysates were saved for testing. In some cases, the culture filtrates and lysates were dialysed in a rocking dialyser against running tap water at 4° for periods from 3 hr. to 4 days and the materials within the bag were saved for testing. The cell-free extracts, culture filtrates, lysates, and dialysis residue were stored at -20° when not in use.

Assay systems for detection of possible lytic principles. In general, organisms grown in nutrient broth for 16 hr. were used to detect lytic activity. The organisms were either kept in their culture medium or were removed by centrifugation and resuspended in the test solutions. In a few tests, 6 and 10 hr. crops of organisms were used. In experiments designed to study tem-

perature effects, 16 hr. crops of organisms either retained in their culture medium or centrifuged and resuspended in M/30 phosphate buffer (pH 8.0) were heated at various temperatures for 10 min. immediately before being used in the assay system. On occasion, assay organisms were grown in the defined medium rather than in nutrient broth and were then either retained in their culture medium or were resuspended in staled nutrient broth. In addition to intact organisms, isolated cell walls were used as an assay tool. The method of cell-wall extraction and purification used was that of Salton & Horne (1951).

RESULTS

Compounds which induced lysis of Bacillus subtilis strain Marburg

Of the 48 compounds listed in Table 2, only 5 were able to induce lysis under the conditions of the experiments (Table 1). The active substances included cysteamine and 4 closely related derivatives; the structural formulae of these compounds are given in Fig. 1. It should be noted that when aminoethyl isothiuronium bromide (AET) and S- γ -aminopropyl isothiuronium bromide

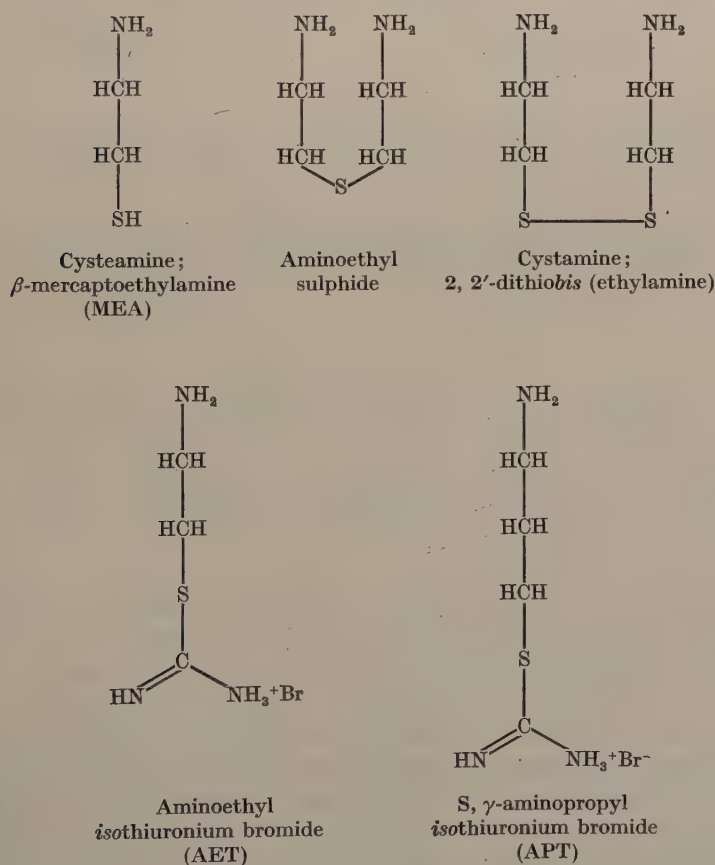


Fig. 1. Structural formulae of the compounds which induce lysis.

(APT) are dissolved in neutral aqueous solutions, an intra-transguanylation occurs so that the active compounds are believed to be 2-mercaptoethylguanidine and 3-mercaptoethylguanidine, respectively (Shapira, Doherty & Burnett, 1957).

The bacteriostatic concentration of each of the cysteamine compounds is indicated in Fig. 2 and the concentrations which induced lysis are presented in Fig. 3. It is apparent that sub-bacteriostatic concentrations were quite

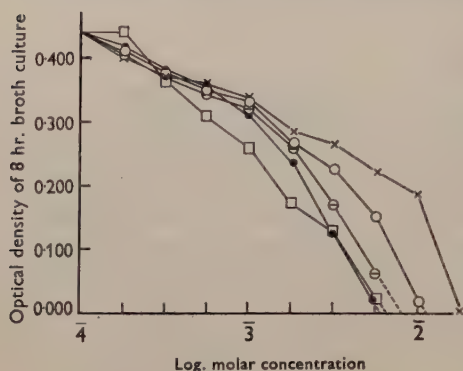


Fig. 2

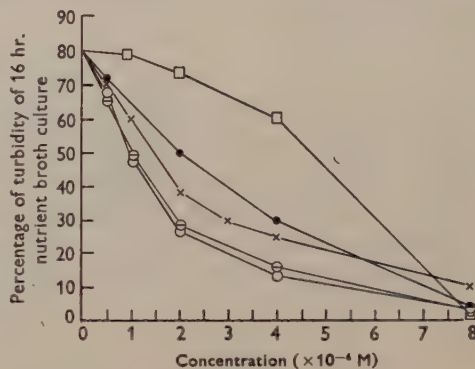


Fig. 3

Fig. 2. Ability of various concentrations of the cysteamine compounds (added at the time of inoculation) to prevent growth of *Bacillus subtilis* strain Marburg in nutrient broth. Growth in the absence of the compounds was equivalent to that obtained in the presence of a concentration of 1×10^{-4} M. \square — \square , APT; \bullet — \bullet , AET; \times — \times , cysteamine; \bigcirc — \bigcirc , cystamine; \bigcirc — \bigcirc , amino ethyl sulphide.

Fig. 3. Ability of sub-bacteriostatic concentrations of the cysteamine compounds (added to 16 hr. nutrient broth cultures of *Bacillus subtilis*) to induce lysis. The readings were made at 24 hr. \square — \square , APT; \bullet — \bullet , AET; \times — \times , cysteamine; \bigcirc — \bigcirc , cystamine; \bigcirc — \bigcirc , amino ethyl sulphide.

effective in induction of lysis. The time required for induction of lysis by various concentrations of cysteamine is given in Fig. 4. It may be noted that lysis occurred mainly between 4 and 8 hr. after the addition of the compound to post-log.-phase cultures. In the presence of suboptimal concentrations of cysteamine, a small amount of lysis occurred in the subsequent 16 hr. period; no additional cysteamine-induced lysis occurred thereafter.

The spectrum of bacterial sensitivity to induction of lysis by cysteamine

Of the test bacterial strains listed in Table 1, it may be observed that the majority of the strains of *Bacillus* especially within the species *Bacillus subtilis*, were susceptible to lytic induction. Attempts were made to induce lysis of strains of other genera by cysteamine by: (1) increasing the concentration of cysteamine to as much as 1×10^{-2} M; (2) adjusting the pH value of post-log.-phase cultures to 8.5; (3) extending the time of contact between cysteamine and the strains to 6 days. These attempts to induce lysis were unsuccessful.

Physical and chemical environmental requirements for induction of lysis of Bacillus subtilis strain Marburg by cysteamine

Despite the fact that sub-bacteriostatic concentrations of cysteamine induced lysis, such concentrations could be included in the culture medium at the time of inoculation without harm to the organisms of the inoculum. The organisms proceeded to complete the lag and logarithmic growth phases

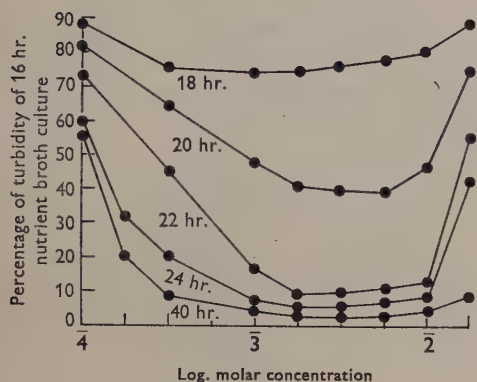


Fig. 4

Fig. 4. Ability of various concentrations of cysteamine (added to 16 hr. nutrient broth cultures) to induce lysis in *Bacillus subtilis*. In the absence of the compound, cultures decreased in turbidity to 70% of the 16 hr. turbidity at 40 hr.

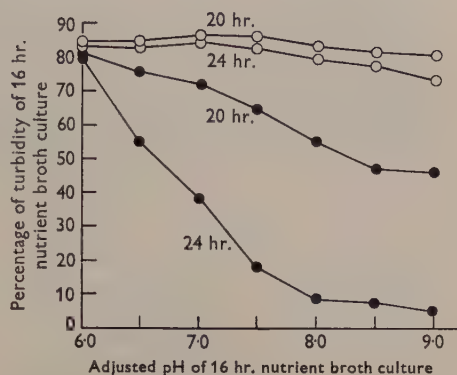


Fig. 5

Fig. 5. Effect of changing the pH value of 16 hr. nutrient broth cultures of *Bacillus subtilis* on the ability of cysteamine (added at the time of the pH adjustment) to induce lysis. The pH value of the 16 hr. cultures before adjustment was 8.7. ○—○, no cysteamine; ●—●, 1×10^{-3} M cysteamine.

of growth as rapidly as organisms in plain nutrient broth. The post-log. phase in each case was reached in approximately 10 hr.; in the cultures containing cysteamine, lysis occurred between 14 and 18 hr. Organisms in the control cultures retained their structural integrity for 6 days, at which time they were discarded. Cysteamine and aminoethyl sulphide could also be added either at the time of inoculation or during the post-log. period to obtain lysis, whereas the two isothiuronium compounds (AET, AEP) induced lysis only when they were added to post-log. phase cultures.

The influence of various physical and chemical environmental factors on induction of lysis by cysteamine is indicated in Table 3 and in Fig. 5. The most efficient conditions for such lysis included growth and maintenance of the cultures in nutrient broth in shaken shallow layers at 37° without alteration of the normal post-log. pH value of 8.7. Because of a deficiency of available manganese in nutrient broth (Weinberg, 1955) such cultures could not sporulate. Under conditions where sporulation occurs, as in nutrient agar, lysis induced by cysteamine required approximately 24–28 hr. rather than 4–8 hr. (Weinberg, 1957). It is probable that germination of the spores followed by

logarithmic growth was required before the organisms became competent to lyse.

Attempts were made to suppress lysis in nutrient broth by increasing the molar strength of the environment with high concentrations of sucrose or glucose. As is indicated in Table 3, high concentrations of these carbohydrates caused both control and cysteamine-exposed organisms to lyse. Lower concentrations retarded the induction of lysis by cysteamine. The formation

Table 3. *Effect of environmental conditions on ability of cysteamine to induce lysis of post-log. phase assay organisms of Bacillus subtilis strain Marburg*

The organisms were grown in shaken shallow layers of nutrient broth (except in E) at 37° for 16 hr. Cysteamine (1×10^{-3} M) was added at 16 hr. and incubation was continued under the various test conditions for a subsequent period of 48 hr.

Condition during 48 hr. post-log. phase		Rapidity and extent of lysis	
		Control organisms	Cysteamine-exposed organisms
(A) Amount of aeration	(1) Shaken shallow layer	None	Rapid, complete
	(2) Aspirated air through deep layer	None	Slow, partial
	(3) Compressed air through deep layer	None	Slow, partial
	(4) Stationary shallow layer	Slow, partial	Slow, complete
(B) Temperature	(1) 80°	None	None
	(2) 58°	Rapid, complete	Rapid, complete
	(3) 37°	None	Rapid, complete
	(4) 25°	None	Slow, complete
	(5) 2°	None	None
(C) pH value (see Figure 5)	(1) 8-9	None	Rapid, complete
	(2) 7-8	None	Slow, partial
	(3) 6-7	None	Slow, slight
(D) Assay organisms grown in nutrient broth	(1) Resuspended in nutrient broth filtrate	None	Rapid, complete
	(2) Resuspended in defined medium filtrate (adjusted to pH 8.0)	None	Slow, partial
	(3) Resuspended in M/30 PO_4 buffer (pH 8.0)	Slow, partial	Slow, complete
(E) Assay organisms grown in defined medium	(1) Resuspended in nutrient broth filtrate	None	Rapid, complete
	(2) Resuspended in defined medium filtrate (adjusted to pH 8.0)	None	None
(F) Increase in molar strength	(1) 1.4 M-sucrose or glucose	Immediate, complete	Immediate, complete
	(2) 0.7 M-sucrose or glucose	Rapid, partial	Rapid, complete
	(3) 0.14 M-sucrose or glucose	None	Slow, complete

of protoplasts was not observed even when bovine serum albumin was added to the lysing preparations that were being examined by phase microscopy.

Of interest are the observations that a few of the 43 compounds which did not induce lysis partially suppressed the activity of cysteamine. The cysteamine-suppressing compounds included D- or L-cysteine, DL-cystine, D-penicillamine, thiazolidine thione and thiazolidine-4-carboxylic acid. The last compound, an antimetabolite of proline, was the most efficient suppressor of cysteamine (Fig. 6); L-proline had no effect on the activity of thiazolidine-4-carboxylic acid.

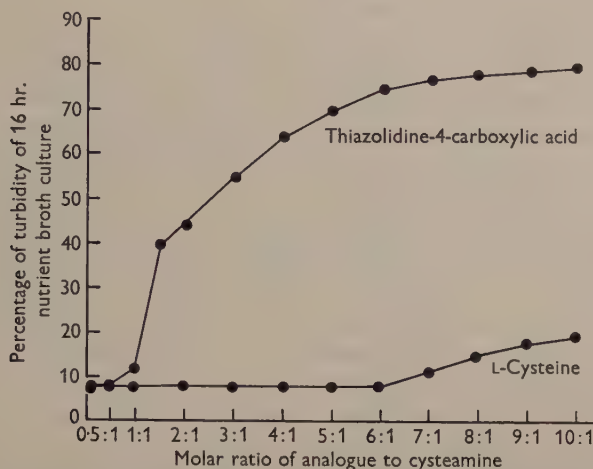


Fig. 6. Ability of thiazolidine-4-carboxylic acid and L-cysteine to suppress lysis-induction activity of cysteamine in *Bacillus subtilis*. The analogue and cysteamine were added at 16 hr. and the readings were made at 24 hr.

Various inorganic ions were tested for their effect on cysteamine-induced lysis. Cu^{++} slightly accelerated lysis; Mg^{++} and MoO_4^{--} were inactive; Fe^{++} , Mn^{++} , and Zn^{++} slightly delayed lysis; Co^{++} and Ni^{++} totally inhibited the cysteamine effect. In each case a concentration equimolar to cysteamine was used; since some of the metallic ions are bacteriostatic in these concentrations, both cysteamine and the ions were added to post-log. cultures rather than to fresh cultures.

Other requirements for cysteamine-induced lysis included: (1) metabolizing organisms rather than organisms inactivated by exposure to 80° for 10 min. or isolated cell walls; (2) the continual presence of cysteamine (or a compound synthesized by cysteamine-exposed organisms) in the culture filtrate. When at any time during the period required for lysis to occur, the cysteamine-exposed organisms were removed from their culture medium and resuspended in normal culture filtrate of the same age, lysis was considerably slower and often incomplete.

Presence of formed elements in the lysate

As lysis proceeded to completion, the majority of organisms (as determined by successive stained preparations) first became Gram-negative and then

disappeared. When organisms in lysing cultures were observed by phase microscopy, the organisms appeared to become less dense before they disappeared. No formed elements were detected in lysates by phase microscopy. Cell-wall stains of partially lysed cultures revealed circular 'ghosts' which were not evident in completely lysed preparations. Similarly, circular ghosts were seen in electron photomicrographs of partially-lysed cultures. Electron photomicrographs of normal cultures contained only intact organisms while those of completely lysed cultures contained no formed elements except flagella. Seemingly completely lysed cultures were, however, not sterile; plate counts to detect viable organisms indicated that a few hundred organisms/ml. lysate survived exposure to cysteamine. It is noteworthy that these few surviving organisms were not able to multiply in filtrates of cysteamine-lysed cultures, whereas survivors of populations lysed by other means (such as high concentrations of sucrose) were usually able to undergo logarithmic growth in their homologous culture media when incubation was continued.

The ability of dialysed lysates, culture filtrates, and fresh nutrient broth to induce lysis (in the absence of cysteamine) of normal and heated assay organisms with and without added Co^{++}

An extensive series of experiments was performed to determine whether cysteamine-exposed organisms produced a bacteriophage or an unusually large quantity of an autolytic enzyme. Tests for bacteriophage production consisted of: (a) adding successive crops of post-log. normal organisms to lysates, and (b) the addition of lysates to normal organisms of various ages in nutrient broth and on nutrient agar. To detect the presence of autolytic enzymes, similar experiments were performed with soluble extracts of normal and cysteamine-exposed crops of organisms of various ages which had been ruptured by sonic oscillation and by Mickle disintegration. No evidence of lytic activity in any of these preparations was obtained except in those containing residual cysteamine. When cysteamine was present in the minimum concentration required for lysis of the first crop of organisms, subsequent crops were not lysed in the particular sample. When a twofold, threefold, fourfold, or fivefold concentration of cysteamine was contained in the original sample, two, three, four or five but not six crops of post-log. organisms, respectively, could be added successively to the sample and be completely lysed.

To remove residual cysteamine from cell-free extracts or from lysates, the samples were dialysed in a rocking dialyser against running tap water at 4° for periods from 3 hr. to 4 days. Samples of culture filtrate and fresh nutrient broth were likewise dialysed for 2 days. Dialysed cell-free extracts possessed no lytic activity for normal post-log. assay organisms but lysates from cysteamine-exposed organisms did possess lytic activity when dialysed for at least 2 days. Normal culture filtrate and fresh nutrient broth which had been dialysed for 2 days similarly acquired lytic activity, although they were less efficient than dialysed lysates. The time required for lysis of 50 % of crops of assay organisms added to the various samples is summarized in Table 4. It may

be seen that as the residual cysteamine was removed from lysates by dialysis up to 24 hr. the lysates became somewhat less active but that activity was significantly greater in lysates dialysed for 48 hr. With some lysates, a period of dialysis of 3 days was required to obtain maximum activity; no additional activity of any sample was gained by a 4th day of dialysis.

Table 4. *Effect of dialysis of test samples and of heat and Co⁺⁺ activation on rapidity of lysis of post-log. phase assay organisms of Bacillus subtilis*

Test samples	Unheated organisms	Organisms exposed to 58° for 10 min.	Unheated organisms exposed to 2×10^{-3} M Co ⁺⁺ during tests
(A) Post-log. phase culture filtrates from which normal organisms were removed			
	Time for 50 % lysis (hr.)		
(1) Non-dialysed	No lysis	1.3	2.4
(2) Non-dialysed plus 8×10^{-4} M-cysteamine	4.0	0.6	6.5
(3) Dialysed 48 hr.	12.0	1.2	3.0
(B) Post-log. phase lysate from cysteamine-exposed cultures			
(1) Non-dialysed (contained slight amount of residual cysteamine)	13.0	1.2	3.0
(2) Dialysed 3 hr.	15.0	1.0	3.0
(3) Dialysed 6 hr.	16.0	1.1	3.0
(4) Dialysed 24 hr.	16.5	1.0	3.0
(5) Dialysed 48 hr.	8.4	0.5	2.6
(C) Fresh nutrient broth			
(1) Non-dialysed	No lysis	No lysis	6.5
(2) Dialysed 48 hr.	24.0	2.1	4.5

Since the autolytic enzyme of post-log.-phase organisms of *Bacillus subtilis* studied by Strange & Dark (1957) was activated by exposure to 58° and by the presence of high concentrations of Co⁺⁺, it seemed of interest to include a study of the effect of these two conditions in lysis of *B. subtilis* by the test samples. Heat activation was accomplished by heating the assay organisms (in a small volume of pH 8.0 phosphate buffer) for 10 min. at 58° before suspension of the organisms in the various test samples. The actual tests were then performed at 37°, although the results were similar when the tests were conducted at 58°. Activation by Co⁺⁺ was tested by adding to the test samples a solution of CoSO₄·7H₂O to obtain a final concentration of Co⁺⁺ of 2×10^{-3} M.

It can be observed in Table 4 that exposure of the normal post-log. phase assay organisms to 58° markedly accelerated lysis in all samples. As was true of non-heated organisms, organisms exposed to cysteamine or to the 48 hr. dialysed lysate lysed most rapidly. The presence of Co⁺⁺ did not accelerate lysis of heated organisms but did significantly hasten the lysis of non-heated organisms with one exception. The single exception was that of the culture filtrate containing cysteamine and it is believed that the addition of Co⁺⁺ to this sample neutralized the cysteamine by forming an inactive chelate. As

evidence of chelate formation, solutions containing cysteamine and Co^{++} developed a pink colour. Lysis eventually did occur in these samples presumably because of the excess Co^{++} .

DISCUSSION

Lytic phenomena in the genus *Bacillus* have been reported quite frequently within the past 3 years (Greenberg & Halvorson, 1955; Ivánovics & Alföldi, 1957; Nomura & Hosoda, 1956; Norris, 1957; Strange & Dark, 1957). The present observations are similar to those of the majority of the previous reports in that (a) post-log. phase rather than younger crops of organisms are most readily lysed, (b) there is considerable strain and species specificity, (c) a complex medium usually is required, and (d) most of the formed elements of the organism are destroyed during lysis.

The present observations differ from those of the previous studies on lysis of organisms of *Bacillus* sp. in that the assay organisms themselves actively participate in the lytic process. Organisms inactivated by heat do not lyse nor are isolated cell walls destroyed by cysteamine. In order to lyse, the assay organisms must be maintained at 37° in staled broth in the presence of the cysteamine compounds for several hours.

Induction of lysis of strains of *Bacillus* spp. by the cysteamine compounds is somewhat comparable to induction of lysis of various bacteria of different genera by glycine (Cowles, 1946). With glycine, post-log. phase metabolizing assay organisms are required, and 3–20 hr. are needed for the appearance of visible lysis. As with cysteamine, the cultures do not become completely sterile. Although glycine has a much broader bacteriolytic spectrum than has the cysteamine compounds, it is much weaker in activity. Concentrations of glycine as great as molar are required, whereas in the present experiments, active compounds induced lysis optimally at concentrations as low as 10^{-4} M.

An interesting feature of induction of lysis by cysteamine is that post-log. phase organisms which are used as inoculum for fresh cultures do not lyse within 4–8 hr. in the presence of the compound in fresh broth. Rather, the organisms proceed through the normal phases of growth and give rise to progeny which do lyse within 4–8 hr. in the post-log. phase provided cysteamine has not been removed from the environment. Apparently, fresh nutrient broth lacks factors possessed by staled broth that are required for cysteamine lysis. An alternative hypothesis is that fresh broth permits the post-log. phase organisms to begin logarithmic growth before 4 hr. has elapsed and they thus temporarily escape the usual fate of post-log. phase organisms exposed to cysteamine.

The requirement for nutrient broth rather than the defined medium for the exhibition of cysteamine activity is analogous to the situation reported by Ivánovics & Alföldi (1957) in which induction of lysis of *Bacillus megaterium* by thioglycollate or ultraviolet light occurred only when the organisms were grown in a complex medium. Perhaps cell walls are synthesized differently in complex media and simpler defined nutritive environments (cf. Litwac & Pramer, 1956). In the present study, however, organisms grown in the

defined medium did lyse in the presence of cysteamine when they were suspended in staled nutrient broth. It is of interest to note that the intra-transguanylation product of AET (i.e. 2-mercaptoethylguanidine) was found to be considerably more active in protecting bone marrow cells from X-irradiation when the cells were suspended in plasma rather than in a chemically defined medium or in saline (Smith, 1957).

Of interest are reports that cystamine is metabolized to a dark coloured compound by extracts of mammalian amine oxidases (Cavallini, De Marco & Mondovi, 1957; Bergeret & Blaschko, 1957). In the present study, one of the sensitive and two of the resistant strains of *Bacillus* spp. likewise produced a dark pigmented compound from cysteamine and from cystamine. Unpublished observations in this laboratory indicate that cysteamine is neither deaminated nor desulphurated by intact organisms or by cell-free extracts of sensitive strains.

Possible mechanisms whereby the cysteamine compounds induce lysis of sensitive strains include the following:

A. *Activation of a normal 'lysozyme-like' autolytic enzyme.* Autolytic enzyme molecules are present in large amount in post-log. phase organisms of *Bacillus* spp. (Richmond, 1957; Strange & Dark, 1957). This enzyme presumably facilitates the emergence of the spore through the vegetative cell wall. In the present study, spores did not develop because of the lack of sufficient Mn^{++} (Weinberg, 1955) but the organisms did possess an autolytic mechanism as demonstrated by their rapid autolysis in the presence of Co^{++} or when heated to 58° . Possible activation of this autolytic mechanism by the cysteamine compounds might be comparable to the activation of egg-white lysozyme by ethylene diamine tetraacetic acid (Repaske, 1956).

B. *Suppression of new cell-wall synthesis.* If continual synthesis of new cell-wall material by post-log. phase organisms is necessary to balance the normal autolytic mechanism, then a suppressor of such synthesis would indirectly cause the organisms to lyse. It is known that cell-wall synthesis may be suppressed by (a) penicillin and (b) a deficiency of a constituent of cell walls such as α, ϵ -diaminopimelic acid in the nutrient for certain auxotrophs (McQuillen, 1958). Unlike penicillin, cysteamine and related compounds are not active during logarithmic growth; moreover, Co^{++} neutralizes rather than enhances these compounds, presumably by formation of an inactive chelate. Conceivably, such compounds, rather than an unknown analogue, might be incorporated into cell walls of post-log. phase organisms of sensitive strains with the result that such walls could not maintain their structural integrity.

C. *Binding of metallic ions in cell walls.* Kozloff & Lute (1957) presented evidence that cell walls of *Escherichia coli* contain zinc atoms. It is possible that metallic ions are present in the walls of *Bacillus* and that post-log. phase organisms depend on such ions to maintain the structural integrity of the walls to a greater extent than do younger cells. However, numerous metal-binding agents other than cysteamine and its derivatives have been found in this laboratory (unpublished observations) to be unable to induce lysis of post-log. phase organisms of *Bacillus* spp.

D. *Binding a metallic ion inhibitor of lysis.* Autolysis of *Streptococcus faecalis* has been observed to be suppressed by Mn^{++} (MacLeod, 1951; van Eys & Pearson, 1954). In the present study, lysates, culture filtrates and fresh nutrient broth gained autolytic activity by being dialysed for 1–2 days. It is conceivable that such dialyses remove metallic ions that are tightly bound to non-dialysable molecules in the preparations, and that such metallic ions are suppressors of autolytic activity of post-log. phase organisms of *Bacillus*. Thus, dialysed preparations might induce lysis in the absence of, for example, cysteamine, whereas in non-dialysed materials, lysis would occur only if the active compounds herein reported combined with certain metallic ions of these materials.

E. *Suppression of a protease that might inhibit lysis.* Experiments performed in this laboratory (to be published) indicate that cysteamine and its derivatives suppress the auto-protease activity of extracts of sensitive organisms. If protease activity is needed to inhibit normal autolysis of post-log. phase organisms then a suppressor of such activity would indirectly induce lysis. Obviously much additional investigation is needed to establish the relationship, if any, among the biochemical mechanisms whereby cysteamine and similar compounds protect cells against radiation damage, suppress protease activity, and induce lysis of post-log. phase organisms of sensitive strains of *Bacillus*.

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On the Intestinal Yeast Flora of Horses, Sheep, Goats and Swine

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SUMMARY: From the caeca of 252 horses, 503 sheep, 250 goats and 250 swine, 486 yeast isolates belonging to 28 species and 1 variety were obtained. The distribution of the yeasts of any species and for *Candida albicans* respectively was: horses, 52.4 %, 4.4 %; sheep, 6.8 %, 4.2 %; goats, 6.4 %, 0.8 %; swine, 88.8 %, 9.2 %. The suitability of the sheep and goats as hosts for yeasts of any species seems very limited.

The most frequent occurrences for single species were: *Candida slooffii* in swine (48.4 %), *Trichosporon cutaneum* in horses (21.8 %) and *Saccharomyces tellustris* (*Candida bovina*) in swine (14 %).

One isolate of *Cryptococcus neoformans* was obtained from a healthy horse, suggesting that horses might sometimes contribute to the dispersal of this pathogen. Temperature relations, vitamin requirements and some assimilative properties of the following organisms were determined (total number of isolates in brackets): *Saccharomyces tellustris* (3), *Torulopsis glabrata* (6), *T. pintolopesii* (10), *Candida bovina* (6), *C. slooffii* (6), *Cryptococcus neoformans* (23) and *Trichosporon cutaneum* (12).

In an earlier report (van Uden & Carmo Sousa, 1957*a*) various aspects of the ecology of yeasts associated with warm-blooded animals were discussed and the results of a survey of yeasts from cattle intestines presented; now the results of a similar study of the intestinal yeast flora of horses, sheep, goats and swine are given.

METHODS

Two hundred and fifty-two adult horses (including an unrecorded number of mules), 503 adult sheep, 250 adult goats and 250 swine were sampled in the abattoir of Lisbon.

Yeasts were isolated from the caecal contents as described earlier (van Uden & Carmo Sousa, 1957*a*). However, the solid medium used for plating out was adjusted to pH 4.5 and not to pH 3.5, as it was observed that some isolates grow badly or not at all at the latter pH value. As happened in our cattle series filamentous fungi grew in most primary culture tubes, making difficult the yeast isolations. An account of filamentous fungi, isolated from cattle and horses, has been given elsewhere (van Uden & Carmo Sousa, 1957*b*).

The isolated yeasts were classified by the same methods as before (van Uden & Carmo Sousa, 1957*a*). However, whereas the morphological and fermentative properties of all isolates were studied, it was not found necessary for the classification of many isolates to perform assimilation tests with compounds other than D-glucose, D-galactose, sucrose, maltose and lactose. Certain

isolates were studied in more detail and sometimes compared with type strains (obtained from the Centraalbureau voor Schimmelcultures (CBS), Delft, or from the authors of the species) and other strains. This more detailed study included assimilation tests with a number of compounds indicated earlier (van Uden & Carmo Sousa, 1957*a*), sometimes fermentation tests with trehalose, and the determination of temperature relations and vitamin requirements.

Temperature relations. The yeast under study was grown for 24 hr. at 37° in the liquid medium referred to above. A loopful of the suspended growth was then placed in a tube with 5 ml. of the same medium and placed in a temperature-controlled water bath. The degree of growth was noted daily for 5 days. If after this time no growth had occurred the isolate was considered unable to grow at the temperature under study. In this way the maximum and the minimum temperatures were determined; no attempt was made to determine the optimum temperature.

Vitamin requirements. A vitamin-free defined basal medium (Wickerham, 1951) was enriched with various mixtures of vitamins and placed in 5 ml. amounts in test tubes. A complete set of test media was composed of 11 tubes. One tube contained the vitamin-free basal medium only; a second tube contained the basal medium + the following vitamins (calculated/1000 ml.): biotin, 2 µg.; calcium pantothenate, 400 µg.; folic acid, 2 µg.; inositol, 2000 µg.; nicotinic acid, 400 µg.; *p*-aminobenzoic acid, 200 µg.; pyridoxine hydrochloride, 400 µg.; riboflavin, 200 µg.; thiamine hydrochloride, 400 µg.; the nine other tubes contained basal medium + eight of the nine vitamins, one being omitted from each tube.

A loopful of growth from a slope grown for 24 hr. at 37° was suspended in a tube with the vitamin-free basal medium. To exhaust the vitamin reserves of the organisms as far as possible, this tube was incubated for 24 hr. at 37°; then a drop of its content was inoculated into each tube of the complete set of vitamin test media. Incubation was at 37° and growth was noted daily for three weeks.

Temperature is important in vitamin-deficiency tests. As well known from *Neurospora* genetics, a strain may be deficient for a given vitamin only within a given temperature range. The same may be true for yeasts. Kreger-van Rij (1958) actually found that strains of *Candida slooffii* required pantothenate at 37° but not at 30°.

RESULTS

Four hundred and eighty-six isolates belonging to 28 species and one variety were obtained (Table 1). Multiple isolates from any one animal which were found to belong to the same species were counted as a single isolate. In the following account those strains which fitted species descriptions as given by Lodder & Kreger-van Rij (1952) will only be mentioned. Isolates identified with species described after the appearance of Lodder & Kreger-van Rij's monograph and/or submitted to a more extensive study will be considered in more detail.

Table 1. *Yeasts isolated from the caeca of domestic animals*

No. sampled ...	Cattle*	Horses	Sheep	Goats	Swine	
	252	252	503	250	250	
No. with yeasts ...	118 (46.8 %)	132 (52.4 %)	34 (6.8 %)	16 (6.4 %)	222 (88.8 %)	
Organisms						
<i>Candida albicans</i> (Robin) Berkhout	0 (0 %)	11 (4.4 %)	21 (4.2 %)	2 (0.8 %)	23 (9.2 %)	} Obligate saprophytes
<i>C. slooffii</i> van Uden & Carmo Sousa	0 (0 %)	6 (2.4 %)	0 (0 %)	0 (0 %)	121 (48.4 %)	
<i>Saccharomyces tellustri</i> van der Walt (<i>Candida bovina</i>)	1 (0.4 %)	3 (1.2 %)	0 (0 %)	0 (0 %)	35 (14 %)	
<i>Torulopsis pintolopesii</i> van Uden	0 (0 %)	1 (0.4 %)	0 (0 %)	0 (0 %)	0 (0 %)	
<i>T. glabrata</i> (Anderson) Lodder & de Vries	4 (1.6 %)	3 (1.2 %)	0 (0 %)	2 (0.8 %)	8 (3.2 %)	
<i>Candida tropicalis</i> (Castellani) Berkhout	45 (17.9 %)	11 (4.4 %)	3 (0.6 %)	2 (0.8 %)	15 (6 %)	} Facultative saprophytes
<i>C. krusei</i> (Castellani) Berkhout	33 (13.1 %)	21 (8.3 %)	6 (1.2 %)	4 (1.6 %)	37 (14.8 %)	
<i>C. parapsilosis</i> (Ashford) Langeron & Talice	3 (1.2 %)	9 (3.6 %)	3 (0.6 %)	0 (0 %)	2 (0.8 %)	
<i>Trichosporon cutaneum</i> (De Beurman, Gougerot & Vaucher) Ota	5 (2 %)	55 (21.8 %)	0 (0 %)	0 (0 %)	1 (0.4 %)	
<i>Saccharomyces carlsbergensis</i> Hansen	0	5	0	0	1	} Passers-by and yeasts of uncertain ecological position
<i>S. cerevisiae</i> Hansen	12	8	0	4	22	
<i>S. chevalieri</i> Guilliermond	3	0	0	0	0	
<i>S. drosophilae</i> El-Tabey Shehata, Mrak & Phaff	3	0	0	0	0	
<i>S. fragilis</i> Jørgensen	1	0	1	0	0	
<i>S. italicus</i> Castelli	0	0	0	0	2	
<i>S. italicus</i> var. <i>melibiosi</i> van Uden & Assis-Lopes	0	1	0	0	0	
<i>S. microellipsodes</i> Osterwalder	0	0	0	1	0	
<i>Hansenula anomala</i> (Hansen) H. & P. Sydow	0	1	0	0	0	
<i>H. angusta</i> Teunison & Hall	0	0	0	0	1	
<i>Pichia bovis</i> van Uden & Carmo Sousa	1	0	0	0	0	
<i>P. farinosa</i> (Lindner) Hansen	0	1	0	0	1	
<i>P. fermentans</i> Lodder	3	1	0	0	0	
<i>P. membranaefaciens</i> Hansen	3	1	0	0	7	
<i>Nematospora coryli</i> Peglion	0	0	0	1	0	
<i>Debaryomyces hansenii</i> Guilliermond & Péju	0	1	0	0	0	
<i>Cryptococcus diffluens</i> (Zach) Lodder & Kreger-van Rij	1	0	0	0	0	
<i>C. neoformans</i> (Sanfelice) Vuillemin	0	1	0	0	0	
<i>Torulopsis famata</i> (Harrison) Lodder & Kreger-van Rij	0	0	0	0	1	
<i>Candida brumptii</i> Langeron & Guerra	0	1	0	0	0	
<i>C. guilliermondii</i> (Castellani) Langeron & Guerra	0	4	0	0	0	
<i>C. macedoniensis</i> (Castellani & Chalmers) Berkhout	1	0	0	0	0	
<i>C. mycoderma</i> (Reess) Lodder & Kreger- van Rij	0	0	0	0	12	
<i>C. utilis</i> (Henneberg) Lodder & Kreger- van Rij	10	1	0	0	0	
<i>Rhodotorula mucilaginosa</i> (Jørgensen) Harrison	0	0	1	0	0	
Not classified	2	0	0	0	0	

* After van Uden & Carmo Sousa (1957a); included here for comparison. Figures in parentheses represent the % numbers of positive individuals.

Saccharomyces

Six isolates were identified with *Saccharomyces carlsbergensis* Hansen, 34 with *S. cerevisiae* Hansen, 1 with *S. fragilis* Jörgensen, 2 with *S. italicus* Castelli, 1 as *S. microellipsodes* Osterwalder. One isolate from horse was identical with *S. italicus* var. *melibiosi*. This variety was described by van Uden & Assis-Lopes (1957) who isolated it repeatedly from human faeces. *S. italicus* var. *melibiosi* shows a fermentative behaviour unique among naturally occurring yeasts: melibiose and the galactose part of raffinose are fermented, whereas sucrose and the sucrose part of raffinose are not. This is due to the circumstance that *S. italicus* var. *melibiosi* forms α -galactosidase (melibiase) but apparently not β -h-fructosidase (invertase). Dr N. J. W. Kreger-van Rij (personal communication) found, however, that some strains of *S. italicus* var. *melibiosi*, including the strain from horse, are capable of sucrose fermentation under certain not yet clearly defined conditions. Winge & Roberts (1957) and Losada (1957) obtained hybrids with a fermentative behaviour similar to that of *S. italicus* var. *melibiosi*. Some of their hybrids, while lacking genes for the formation of β -h-fructosidase, are nevertheless capable of sucrose fermentation through the action of an α -glucosidase (maltase). The formation of the latter enzyme depends on the presence of any out of certain (but not all) polymeric genes responsible for maltose hydrolysis. In several of the *S. italicus* var. *melibiosi*-like hybrids sucrose fermentation was observed only when the strains had previously been grown in Pilsner wort (which contains maltose), whereas after growth in glucose + yeast water the same strains did not produce gas in sucrose broth. These results suggest the possibility that the formation of the α -glucosidase in question is more easily induced by its substrate maltose than by its substrate sucrose. The facts so far observed do not even exclude the possibility that sucrose, though being a suitable substrate for this enzyme, might have no inducing capacity at all.

Van der Walt (1957) recently described *Saccharomyces tellustris* for an isolate from South African soil. Kreger-van Rij (1958) found this species to be the perfect form of *Candida bovina*, described by ourselves (1957a) for an isolate from a bovine caecum. So far neither Kreger-van Rij (1958) nor the present authors have observed spores in the type strain of *C. bovina*. Thirty-eight yeasts with the same morphological and physiological properties as *C. bovina* were isolated during the present survey. In all but four of the isolates spores were found; these isolates were therefore identified with *Saccharomyces tellustris* van der Walt. Some of the properties of *S. tellustris*, *C. bovina* and related species can be seen from Table 2. For further information see under *Torulopsis pintolopesii*, *C. bovina* and *C. slooffii*.

Other perfect genera

Of each of the following species one or more isolates (number in parentheses) were obtained: *Debaryomyces kloecckeri* Guilliermond & Peju (1), *Pichia farinosa* (Lindner) Hansen (2), *P. fermentans* Lodder (1), *P. membranaefaciens*

Table 2. *Distinctive properties within the 'glabrata-group'*

Strains	Size of cells* (μ)	Pseudo- mycelium	Minimum temperature (° C)	Fermentation of trehalose (gas)	Vitamin requirements at 37°								
					Biotin	Pantothe- nate	Folic acid	Inositol	Niacin	p-amino- benzoic acid	Pyridoxine	Riboflavin	Thiamine
<i>Torulopsis glabrata</i> (6)	2.5 × 4.5	Absent	Good growth at 20°	+	+	-	-	-	+	-	+	-	+
<i>T. pintolopesii</i> (10)	4.5 × 6	Absent	*24°	-	+	+	-	±	+	-	+	-	+
<i>Saccharomyces tellustris</i> (3)	5 × 8	Primitive	Slow growth at 20°	-	+	+	-	±	+	-	+	-	+
<i>Candida bovina</i> (6)	5 × 8	Primitive	Slow growth at 20°	-	+	+	-	±	+	-	+	-	+
<i>C. slooffii</i> (6)	6 × 10	Well developed	*28°	-	+	+	-	+	+	-	+	-	+

Figures in parentheses are the numbers of strains studied, including the respective type strain.

* These are average figures.

Hansen (8), *Hansenula anomala* (Hansen) H. & P. Sydow (1), *Hansenula angusta* Teunison & Hall (1), *Nematospora coryli* Peglion (1).

Cryptococcus

One isolate from horse was identified with *Cryptococcus neoformans*. To test its pathogenicity, about six million cells were injected intravenously into each of two adult white mice. The animals died after 14 and 24 days respectively. Cultures from liver, kidney, spleen, lung and brain were positive; on histopathological examination numerous lesions with encapsulated yeast cells were found in these organs. A detailed account of these findings will be given elsewhere (van Uden, Braço Forte & Carmo Sousa, 1958). The isolate from horse was studied, together with the type strain and 22 isolates of *C. neoformans* received from other laboratories, with regard to carbohydrate assimilation, vitamin requirements and maximum temperature for growth. The following results were obtained. (i) All strains utilized: D-glucose, D-galactose, sucrose, maltose, D-xylose, D-mannitol, D-sorbitol, inositol; most strains utilized: raffinose, L-arabinose, ethanol, soluble starch; glycerol, inulin weakly or not at all. Lactose and potassium nitrate are not assimilated. (ii) All strains require thiamine but grow well in the absence of other vitamins. (iii) Most isolates had their maximum temperature between 39° and 40°; a few isolates still showed some growth at 40°. It would seem from these results that ability for growth up to 39° and a requirement for thiamine are characteristics which hold for all strains of *C. neoformans* and may be helpful in the identification of this pathogen.

Torulopsis

Thirteen isolates were found identical with *Torulopsis glabrata* (Anderson) Lodder & de Vries. One isolate was identified with the thermophilic species *T. pintolopesii* van Uden. *T. pintolopesii* has the same fermentation and assimilation pattern as *Candida bovina* (*Saccharomyces tellustris*), *C. slooffii* and

(excepting trehalose fermentation) *T. glabrata* and it is felt that these four species should be placed in the same genus. This, however, cannot be done without upsetting the generic framework of the system of classification of the imperfect yeasts as devised by Lodder & Kreger-van Rij (1952). As this system is highly workable and now in general use, we think it advisable that generic modifications be postponed until information has accumulated permitting the reclassification on natural lines of the imperfect yeasts as a whole. Attention is called to the serological work of Jonsen (1955) with several *Candida* species which shows the existence of three clearly distinct serological groups within the genus *Candida*, represented by *C. albicans*, *C. krusei* and *C. pseudotropicalis*, respectively. Extensive studies of this kind may be needed before a natural classification of the imperfect yeasts can be seriously attempted. Meanwhile, we had better leave *Torulopsis glabrata* and *T. pintolopesii* and their relatives *C. bovina* and *C. slooffii* in the genera in which they had to be placed following Lodder & Kreger-van Rij's criteria. For practical purposes we have called these four species the glabrata group (van Uden & Carmo Sousa, 1957c); some of their properties can be seen from Table 2, which shows that *Torulopsis pintolopesii* can be differentiated from *T. glabrata* by its bigger cells, its temperature relations, its vitamin requirements and its inability to ferment trehalose. One isolate was identified with *T. famata* (Harrison) Lodder & Kreger-van Rij.

Candida

Fifty-seven isolates were identified with *Candida albicans* (Robin) Berkhout, 1 with *C. brumptii* Langeron & Guerra, 4 with *C. guilliermondii* (Castellani) Berkhout, 14 with *C. parapsilosis* (Ashford) Langeron & Talice, 31 with *C. tropicalis* (Castellani) Berkhout, 68 with *C. krusei* (Castellani) Berkhout, 1 with *C. utilis* (Henneberg) Lodder & Kreger-van Rij and 12 with *C. mycoderma* (Reess) Lodder & Kreger-van Rij. Thirty-eight isolates were found similar to *C. bovina* van Uden & Carmo Sousa; as indicated above, all but 4 of these isolates formed spores and were therefore classified as *Saccharomyces tellustris*. *C. bovina* is rather similar to *Torulopsis pintolopesii* (Table 2). The slight difference in cell size is not helpful for differentiation in practice. Neither are the differences in vitamin deficiency pattern. Clear differences are the ability of *C. bovina* to form a primitive pseudomycelium in corn meal agar at 37° and to grow below 24°. We have not yet determined the minimum temperature of *C. bovina*, but all strains so far studied grow slowly at 20°. Were it not for the generic separation of *C. bovina* from *Torulopsis pintolopesii*, which for reasons pointed out above will have to be maintained provisionally, we would classify *C. bovina* as a variety of *T. pintolopesii* rather than a separate species. Should the perfect form of *T. pintolopesii* be found it should be considered in our opinion as a variety of *Saccharomyces tellustris*. Kreger-van Rij (1958) recently found spores in a strain of *T. pintolopesii*, but did not define its taxonomical position.

Six isolates from horse could not be identified with any known species. They were described as *Candida slooffii* in a separate paper (van Uden & Carmo Sousa, 1957c); 127 isolates from swine were found identical with this species.

C. slooffii clearly belongs to the glabrata group (Table 2). It can easily be separated from *Torulopsis pintolopesii* and *C. bovina* by its bigger cells, the formation of abundant and well-differentiated pseudomycelium and its high minimum temperature.

Trichosporon

Fifty-six strains were identified with *Trichosporon cutaneum* (De Beurman, Gougerot & Vaucher) Ota as described by Lodder & Kreger-van Rij. Twelve strains were taken at random and studied in more detail (Table 3). The five strains of *T. cutaneum* isolated from our cattle series (van Uden & Carmo Sousa, 1957*a*) had fallen into two distinct groups, one assimilating D-mannitol, D-sorbitol and inositol but not soluble starch, the other assimilating the latter compound but not the former ones. It was suspected, therefore, that *T. cutaneum* as defined by Lodder & Kreger-van Rij might comprise more than one taxonomic entity. The results from the 12 isolates from horse, however, did not substantiate this suspicion, as no clear-cut assimilative groups appeared with regard to the above-mentioned compounds.

Table 3. *Assimilation reactions and vitamin requirements of 12 isolates of Trichosporon cutaneum taken at random from a group of 55*

All 12 isolates assimilated: D-glucose, D-galactose, sucrose, maltose, lactose, L-arabinose, D-xylose and ethanol. Raffinose and inulin were not assimilated by any of the 12 isolates. None of these 12 isolates require any of the following vitamins as nutrients: pantothenate, folic acid, inositol, nicotinic acid, *p*-aminobenzoic acid, pyridoxine or riboflavin.

Isolates	Glycerol	D-mannitol	D-sorbitol	Inositol	Thiamine	Soluble starch	Biotin
128/3110	±	—	—	—	d	±	d
132/711	±	±	±	±	d	±	d
123/3110	±	±	—	—	d	±	d
51/910	±	±	±	±	d	±	d
47/129	±	±	±	±	d	±	d
776/2410	±	±	±	±	d	±	d
119/711	+	+	±	+	d	±	d*
32/910	±	—	—	—	d	±	d
61/129	+	+	+	+	d	+	d*
49/910	+	+	+	+	d	+	d*
88a/2410	+	+	+	+	d	+	d*
62/811	+	±	±	+	d	+	d*

+ = assimilation strongly or moderately positive; ± = assimilation weakly positive; — = assimilation negative; d = deficient (no growth after three weeks' incubation); d* = partially deficient (beginning growth after 1 week's incubation).

Rhodotorula

Only one isolate of *Rhodotorula mucilaginosa* (Jørgensen) Harrison was isolated from one sheep.

DISCUSSION

The distribution of yeasts in the faeces of the various animal groups studied varied greatly. The percentage of positive individuals in the horse group (52.4%) is of the same order as the numbers found by di Menna (1954) in man (46%) and by van Uden & Carmo Sousa (1957*a*) in cattle (46.8%). The

extremely high percentage of yeast-harboured individuals in the group of swine (88.8%) was due to the high prevalence of the obligate saprophyte *Candida slooffii*; if this organism is not considered, the faecal yeast incidence in the swine studied becomes 40.4%, which is of the same order as the figures found in the other animal groups indicated above. On the contrary, the distribution found in sheep (6.8%) and goats (6.4%) were very low; this suggests that neither sheep nor goats are important natural hosts for yeasts.

Yeasts which can be isolated from the intestinal tract of warm-blooded animals fall into three different ecological groups (van Uden & Carmo Sousa, 1957a): (a) obligate saprophytes of warm-blooded animals; (b) facultative saprophytes of warm-blooded animals; (c) passers-by. (A similar division was earlier given by di Menna, 1954.)

Within the group of obligate saprophytes of warm-blooded animals, i.e. yeasts which have their natural habitat in the digestive tract, *Candida albicans* has a special place. This is because it may grow abundantly under certain conditions of the host (for example in man: diabetes, debilitating disease, pregnancy, treatment with broad spectrum antibiotics, etc.) and cause disease ('moniliasis'). Certain yeasts nearly related to *C. albicans* (*C. tropicalis*, *C. stellatoidea*) show a similar behaviour but to a much smaller degree. Moniliasis caused by *C. albicans* apparently occurs with appreciable incidence only in those warm-blooded animals which constitute suitable hosts for this yeast as a saprophyte. One may suspect that there are various degrees of suitability for constituting a host for *C. albicans*, not only from species to species but also from individual to individual within the same species. And one may postulate that the higher the suitability of a given species to be a host for *C. albicans*, the more individuals of this species will actually harbour *C. albicans* and the higher will be the incidence of moniliasis. Further, the higher the suitability of a given individual to harbour *C. albicans* as a saprophyte, the more easily will he develop moniliasis. Some evidence supporting these views is constituted by the following observations. Moniliasis is a relatively frequent disease of man and fowls, two host species with the highest saprophytic incidence of *C. albicans* so far recorded. Meleiro de Sousa & van Uden (unpublished results) found *C. albicans* in the faeces of about 25% of unselected women; in women suffering from vaginal moniliasis the faecal incidence of *C. albicans* was about 75%. Van Uden & Carmo Sousa (1957a) did not find *C. albicans* in the caecal contents of 252 cattle, animals in which albicans-moniliasis seems to be extremely rare. We isolated *C. albicans* from 4.4% of 252 horses, 4.2% of 503 sheep, 0.8% of 250 goats and 9.2% of 250 swine. These frequencies are much lower than those found in man (31%) by di Menna (1954) and fowls (21%) by Jordan (1953).

The glabrata group of yeasts (*Torulopsis glabrata*, *T. pintolopesii*, *Candida bovina*, *C. slooffii*) equally fall into the category of obligate saprophytes. With regard to *T. glabrata* some reserve is proper, since recently Dr J. P. van der Walt (personal communication) isolated a number of strains from South African soil and Dr B. Ranganathan (personal communication) in India from sea water. On the other hand, Wickerham (1957) reported the apparent increase of

human infections by *T. glabrata*. The incidence of *T. glabrata* in the present series is of the same low order as found in man by di Menna (1954) and in cattle by van Uden & Carmo Sousa (1957a).

Torulopsis pintolopesii was originally isolated from mice (van Uden, 1952). Since then we have received a number of isolates similar to *T. pintolopesii* from other workers who isolated them from various sources: turkeys (Ainsworth & Austwick, 1955); rats (Dr J. N. Parle, personal communication from Dr M. E. di Menna; Aschner, Halevy & Awram 1954); mice (Artagaveytia-Allende, 1953; Dr N. Orie, personal communication from Dr N. J. W. Kreger-van Rij); pigeons (Dr C. W. Emmons, personal communication); soil (Dr C. W. Emmons, personal communication). On close examination it was found that some of these isolates belonged to *T. pintolopesii* and others to *Saccharomyces tellustris* or *Candida bovina* (Table 2). Similar strains were isolated by Emmons (1950) from 60 specimens of house mouse (*Mus musculus*) 43 of the brown rat (*Rattus norvegicus*) and 2 of the meadow vole (*Microtus* sp.). It would seem from all this that the natural habitat of *T. pintolopesii* and *C. bovina* (*Saccharomyces tellustris*) is in the intestinal tract of small rodents (mice, rats) and possibly certain birds (turkeys, pigeons). None of these species seems well equipped (no growth or poor growth below 24°, nutritional fastidiousness) for growth outside the intestinal tract. However, Dr Emmons feels sure (personal communication) that he has isolated *T. pintolopesii* or a nearly related yeast repeatedly from soil by passage through the peritoneal cavity of white mice. This suggests that vegetative cells of either species may survive for some time outside the animal body. The finding that many isolates (*Saccharomyces tellustris*) of *Candida bovina* sporulate implies that this species may persist in soil as resting spores. The isolation of only one strain of *C. bovina*, two strains of *Saccharomyces tellustris* and one of *Torulopsis pintolopesii* from 252 horses suggests that the equine intestinal tract is not a suitable habitat for these yeasts; they probably occurred as passers-by and came from food contaminated by mice or rats. Neither does an incidence of 2.5% found for *C. slooffii* point to horses as principal hosts for this thermophilic and fastidious species. However, the incidence of 48.4% for *C. slooffii* found in swine constitutes strong evidence that swine are the natural host for *C. slooffii*; quantitative studies on the swine *C. slooffii* relationship are in progress. *Saccharomyces tellustris* (*C. bovina*) was refound in swine with the rather high incidence of 14%, which points to swine as another natural host for this yeast.

The group of facultative saprophytes of warm-blooded animals, i.e. yeasts which grow naturally both inside and outside the digestive tract, is represented in these series by *Candida tropicalis*, *C. krusei* and *C. parapsilosis*. *Trichosporon cutaneum* belongs to the same group and was found in horses with the surprisingly high incidence of 21.8%. Actually this species was the one most frequently isolated from horses. Not much is known about the ecology of *T. cutaneum*. Of 18 isolates of known origin studied by Lodder & Kreger-van Rij (1952) 9 were of human origin and the other 9 had come from various non-animal substrata (wood-pulp, orange peel, sewage). We isolated this species on some occasions from faeces of man, rabbit and mouse and found

a low incidence of this species in cattle (van Uden & Carmo Sousa, 1957a). *T. cutaneum* has been described under many different names, 54 of which are listed as synonyms by Lodder & Kreger-van Rij (1952). Several of those synonyms have been used to name isolates from human diseases, specially of the skin and hair (references in Conant, Smith, Baker, Callaway & Martin 1954; Reiersöl, 1955). Further study is necessary for an evaluation of the significance of the high incidence of *T. cutaneum* in the intestinal tract of the horse.

Of the isolates which probably belong to the group of passers-by (*Saccharomyces cerevisiae*, *S. carlsbergensis* etc.) *Cryptococcus neoformans* deserves special attention. This species, which causes often fatal diseases of man and warm-blooded animals (see Littman & Zimmerman, 1956) has its natural habitat outside the animal body (soil, pigeon nests) as was shown by Emmons (1951, 1955) and Ajello (1956). Our isolate seems to be the first one to be reported from the intestinal contents of a healthy warm-blooded animal. It suggests the possibility that sometimes horses or other animals may be vectors of *C. neoformans*, aiding in the dispersal of this pathogen.

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The Morphology of *Leptotrichia* Species

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SUMMARY: The morphology is described of two species of filamentous oral bacteria, both of which have in the past been accorded the same name, *Leptotrichia buccalis*. Although these have certain characters in common, it is concluded that they are entirely distinct. Both show signs of a residual life-cycle, suggesting a remote relationship with more complex saprophytes.

The specific name *Leptotrichia buccalis* was proposed by Trevisan (1879) to replace *Leptothrix buccalis* (Robin, 1853) on the grounds that the usage of *Leptothrix*, originally signifying filamentous iron bacteria (Kützing, 1843), had become unworkably confused. The early descriptions of the species were clearly recognizable, but this generic name became in its turn corrupted by application to any micro-organism capable of growing in culture in the form of unbranched filaments, so that even sporing bacilli (Gifford, 1920) have been assigned to it, irrespective of origin, whereas true *Leptotrichia* are exclusively oral parasites and have a very characteristic morphology, so characteristic indeed as to be clearly recognizable in the drawings of Antonj van Leeuwenhoek. The descriptions of Robin (1853), Trevisan (1879) and Goadby (1903), especially the latter, make it clear that the name correctly refers to the Gram-positive, fusiform, anaerobic organism which forms so large an element in smears of the *materia alba* of the teeth.

Confusion has arisen especially in two respects, by the failure to distinguish between *Leptotrichia buccalis* and *Fusobacterium* species (Hine & Berry, 1937; Omata & Disraely, 1956), and by application of the name to the second type of micro-organism described in this paper, a filamentous branched aerobe (Bulleid, 1925; Bibby & Berry, 1939; Ludwig, 1955). For this species, the name *L. dentium* has been proposed (Davis & Baird-Parker, 1958). The purpose of this paper is to describe its morphology and cytology, to compare it with *L. buccalis* and to suggest their possible relationships with one another and with other bacterial groups.

MATERIALS

Strains of *Leptotrichia buccalis* were isolated in McIntosh and Fildes jars containing an atmosphere of 90 % (v/v) hydrogen + 10 % (v/v) carbon dioxide. The medium employed contained (% w/v) proteose peptone (Difco), 1; yeast extract (Oxoid), 0.1; Lab-Lemco, 0.3; glucose, 1; soluble starch (Analar), 0.2; L-cysteine hydrochloride, 0.05; anhydrous Na₂HPO₄, 0.5; agar, 1.5; dissolved in distilled water. This medium was autoclaved at 10 lb./sq.in. for 20 min. After autoclaving the following additions were made: 5 % (v/v) Seitz-filtered serum; sulphathiazole to 0.05 mg./ml.; ethyl violet to a final concentration of 1:15,000.

The pH of the medium after autoclaving was 7.6. Plates were poured immediately before use, and inoculated with saliva or *materia alba*. Colonies appeared after 2–3 days of incubation at 37°. Isolated colonies were replated on the same medium with the omission of the ethyl violet and sulphathiazole.

Strains of *Leptotrichia dentium* were isolated on a medium containing (% w/v): peptone (Oxoid), 1; yeast extract (Oxoid), 0.3; soluble starch (Analar), 0.2; sodium chloride, 0.5; agar, 1.5; in beef heart broth and adjusted to pH 7.6. The medium was autoclaved at 10 lb./sq.in. for 20 min. Plates of this medium were poured at c. 45° and inoculated with 1 ml. of a suspension of *materia alba* well dispersed in normal saline, and incubated for 2–3 days aerobically at 37°. Submerged colonies of *L. dentium* were removed by cutting out a small piece of agar and used to inoculate further poured plates. Surface plating was used when a reasonably pure inoculum had been obtained.

Morphological studies were made upon material stained for cell walls by the method of Hale (1953) in the case of *Leptotrichia dentium*, and by tannic-acid and crystal violet (Robinow, 1945) for *L. buccalis*, which gave better results by this method. Colonies were photographed *in situ*.

RESULTS

The colonies of *Leptotrichia buccalis* shown in Pl. 1, figs. 1 and 2 are of a mature (3-day) and a growing (24 hr.) colony, respectively; the 'Medusa-head' appearance is characteristic. By comparison, the colonies of *L. dentium* more closely resembled those of actinomycetes. The submerged colonies of *L. dentium* on primary isolation had a tangled appearance (Pl. 1, fig. 3) and all growth under these microaerophilic conditions was thin and sparse (Pl. 1, fig. 4). Aerobic surface colonies grew much more profusely (Pl. 1, fig. 5), but in all cases the edge consisted of outgrowing, branched filaments.

On first isolation the constituent elements of the colonies of *Leptotrichia buccalis* were fusiform, with very occasional small branches (Pl. 1, fig. 6). At later stages of culture, filaments with side-branches were sometimes found (Pl. 1, fig. 7), but these were never common, and the branches were of the impermanent type, rapidly separated by a septum. L-Form production was very common in these cultures. In the initial stage of L-form production a section of filament showed signs of spiral twisting (Pl. 1, fig. 8), and then swelled into a large, almost globular structure, from which presumed L-forms were released (Pl. 1, fig. 9).

In *Leptotrichia dentium* branching was frequent at most stages of culture, and could, as already noted, be observed at the edges of the colonies (Pl. 1, fig. 4; Pl. 2, fig. 10). This species not only shows normal dichotomous branching of this type, but reproduces by branching in a very characteristic manner; this is illustrated in Pl. 2, fig. 11, and develops in the following manner (Fig. 1): a filament becomes segmented (*a*, *b*) and proceeds to break at the points of division, in a zig-zag manner (*c*). At the breaks, much narrower filaments grow outwards (*d*, *e*), and eventually the sections separate, each consisting of a short filament with a wider and a narrower portion (*f*). These 'whip-handles' are

diagnostic of *L. dentium* and may be seen in the majority of figures in Pl. 2. As the thinner parts of the filaments extend the diameter increases until the original diameter is restored (Pl. 2, figs. 12, 13). The wide filaments also reproduce by breaking down into spore-like bodies, in a manner strongly reminiscent of the sporogenous filaments of *Streptomyces* (Pl. 2, figs. 14, 15). These 'spores' germinate by a tube, and one is visible in Pl. 2, fig. 12 (arrowed). As in the case of *Leptotrichia buccalis*, *L. dentium* readily yields apparent L-forms, and in this case also the first sign is a spiral formation of the filaments (Pl. 2, fig. 16) followed by the production of swollen bodies (Pl. 2, fig. 17).

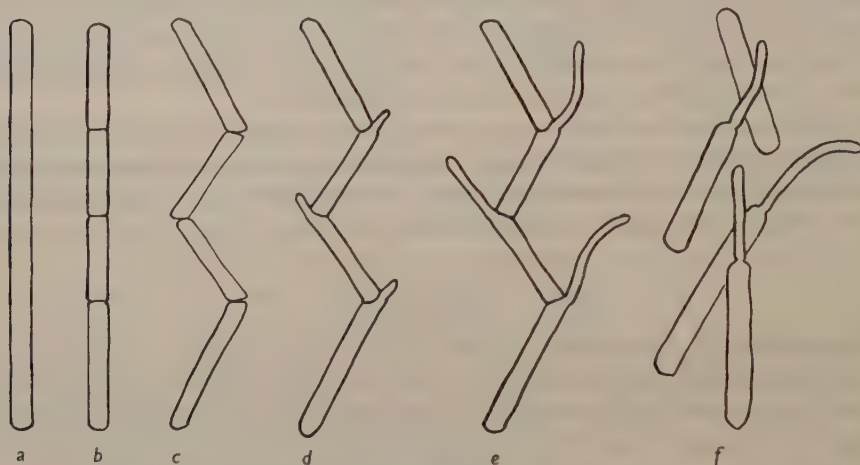


Fig. 1. Diagrammatic representation of a common reproduction process in *Leptotrichia dentium*. (a) An aseptate thick filament. (b) Regular septation of filament. (c) Filament dividing at septa to adopt zig-zag form reminiscent of 'post-fission movements'. (d) Germination of segments at point of previous contact. (e) and (f) Growth of germination tubes and separation of typical 'whip-handle' forms.

DISCUSSION

The morphology of *Leptotrichia buccalis* and *L. dentium* (previously described as *L. buccalis*) is shown to be distinct, although resemblances exist. *L. buccalis* branches very sparingly whereas *L. dentium* does so freely, and also retains the potentiality to form occasional chains of spores. In addition to these Streptomyces-like characters, the curious alternation between thicker and thinner filaments, the latter growing out almost like germination tubes from the former, is known to occur in Streptomyces. Very similar appearances have been described from submerged cultures of Streptomyces by Neukirch (1902) and by Péneau, Hagemann, Velu & Peyré (1954). The *Vierhyphensporen* of Lieske (1921), also bear a close resemblance in general form. Bisset (1957) showed that the vegetative mycelium of Streptomyces was capable of producing forms reminiscent of Micromonospora, Actinomyces and Nocardia. Upon this evidence the suggestion was made that the parasitic actinomycetes may have evolved by a degenerative process from Streptomyces, or some common ancestor. It is possible that the two species of *Leptotrichia* described

in this study may also represent degenerate forms of actinomycetes. How close the relationship between *Leptotrichia buccalis* and *L. dentium* may be is a matter of doubt. Almost certainly, if they are related, the former is an even more degenerate parasitic form than the latter, having become anaerobic and lost almost entirely its power of branching.

We wish to thank Dr K. A. Bisset, for advice and much valuable discussion relating to this work.

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EXPLANATION OF PLATES

PLATE I

- Fig. 1. *Leptotrichia buccalis*, 3-day colony on agar; $\times 16$.
- Fig. 2. As fig. 1 at 24 hr.; $\times 32$.
- Fig. 3. *L. dentium*, submerged colony on primary isolation pour plate, 3 days old; $\times 16$.
- Fig. 4. *L. dentium*, anaerobic surface colony, 3 days old. $\times 32$.
- Fig. 5. *L. dentium*, aerobic 2-day colony on agar surface; $\times 16$.
- Figs. 6-9. *L. buccalis*, stained tannic acid/crystal violet; $\times 1600$.
- Fig. 6. Typical cell forms and example of apical crutch formation.
- Fig. 7. Filament bearing impermanent lateral branches.
- Fig. 8. Filament showing spiral form in early stage of L-cycle.
- Fig. 9. Presumed large-body formation in later stage of L-cycle.

PLATE 2

Figs. 10-17. *L. dentium*, stained phosphomolybdic acid/methyl green. Figs. 11 and 17 from broth cultures, the rest from agar; $\times 1600$.

Fig. 10. Filament showing variation in diameter and permanent branching.

Fig. 11. Germination of elements formed by segmentation of filament (see fig. 1).

Figs. 12, 13. Typical 'whip-handle' forms showing variation in diameter of filaments.

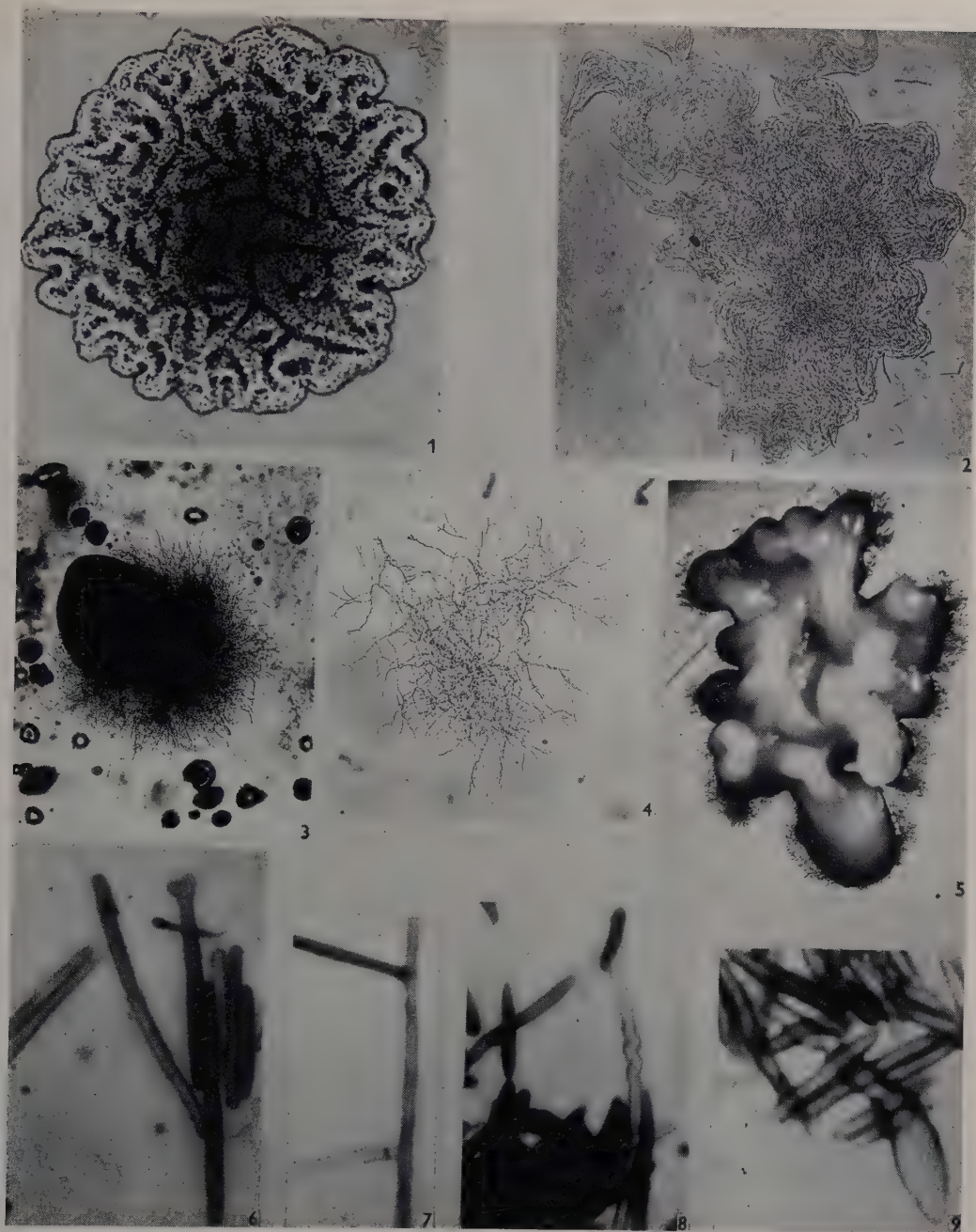
Fig. 14. Streptomyces-like chain of spores.

Fig. 15. Group of presumed spores including one showing germination tube.

Fig. 16. Twisting of filaments in early stage of L-cycle.

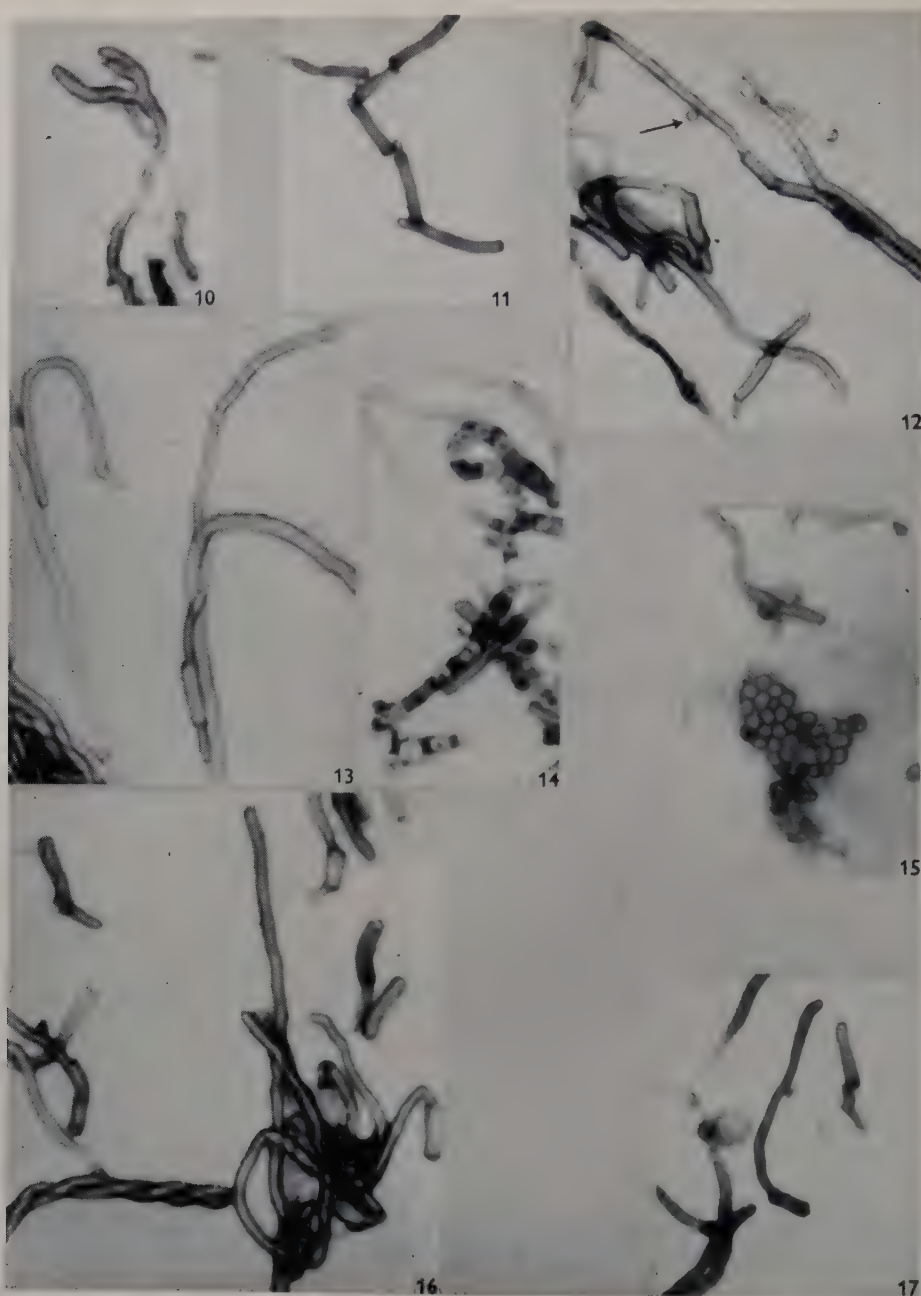
Fig. 17. Presumed large body stage of L-cycle.

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A. C. BAIRD-PARKER & G. H. G. DAVIS—MORPHOLOGY OF *LEPTOTRICHIA* SPP.
PLATE 1

(Facing p. 450)



A. C. BAIRD-PARKER & G. H. G. DAVIS—MORPHOLOGY OF *LEPTOTRICHIA* SPP.
PLATE 2

TAVERNE, J., MARSHALL, J. H. & FULTON, F. (1958). *J. gen. Microbiol.* **19**, 451-461

The Purification and Concentration of Viruses and Virus Soluble Antigens on Calcium Phosphate

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SUMMARY: Chromatography on columns of calcium phosphate has been used to purify and concentrate viruses and their related soluble antigens. The method is an adaptation of one which has already proved successful with proteins and other large molecules. An important advantage is that all operations are carried out at pH 7, elution being effected by alterations in phosphate concentration. The PR8 strain of influenza was used as a model. Chromatography on a calcium phosphate column effected a 30- to 100-fold increase in purity, with a recovery of 50-80 %. By chromatography on a second small column a 10- to 30-fold concentration could be obtained; little further purification occurred. Material with a purity up to 10^6 haemagglutination units/mg. protein was obtained; this compares favourably with material purified by other methods. PR8 soluble antigen was similarly purified and concentrated, and Semliki forest virus was also purified. In preliminary experiments, purification of vaccinia, encephalomyocarditis, Coxsackie and poliomyelitis type III viruses has also been effected. The method is simple and rapid and can be used with crude virus suspensions from different sources. The viruses retained both their infectivity and their antigenicity as judged by complement fixation.

When virus suspensions or their related soluble antigens are required as reagents in a complement-fixation test it is a frequent experience that although complement fixation can be demonstrated in the presence of specific antisera, the amount of the antigen is so low that all thought of sophisticated analyses based on maxima points must be dismissed as impracticable. Sometimes the size of the antigen particle is large enough to allow concentration by centrifugation, but even then the particles often become so aggregated that the degree of concentration is much less than expected. Also, the concentration of tissue fragments with a comparable sedimentation constant usually raises the anticomplementary level of the antigen to such a degree that little improvement of the test results. We have tried to find a simple method of purification and concentration which would be of general application to viruses and their related soluble antigens, and did not degrade the antigens. We have aimed to achieve a concentration of the order of tenfold; more specifically, to obtain an antigen which was not anticomplementary when undiluted and which would fix at least ten units of complement maximally. A method which seems to fulfil these conditions, chromatography on columns of calcium phosphate, is described in this paper.

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Calcium phosphate has been used by many workers to purify and concentrate viruses. In 1941 Salk described a method for the purification of influenza virus elementary bodies in which a precipitate of calcium phosphate was formed in the virus suspension, the precipitate separated, and the virus recovered from it by dissolving the calcium phosphate in a solution of sodium citrate. Stanley (1945) also used this method for the purification of influenza virus, while Elford *et al.* (1948) used adsorption on calcium phosphate in the purification of a number of other viruses.

We have taken advantage of the more recent work of Tiselius and his co-workers (1954, 1955, 1956), who have used columns of calcium phosphate for the chromatographic purification of a number of proteins and also of tobacco mosaic virus. The use of columns of calcium phosphate gives much greater reproducibility and resolving power than the earlier adsorption methods, and Tiselius's method has been extended to deoxyribonucleic acids (Main & Cole, 1957), ribonucleic acids (Semenza, 1957) and hormones (Steelman, 1958), but not, so far as we are aware at present, to animal viruses or their soluble antigens.

Most of the work reported in this paper was carried out with the PR8 strain of influenza A virus grown in embryonated eggs and with its related soluble antigen derived from the infected chorio-allantoic membranes; also with the Semliki forest virus obtained from the brains of mice dying after intracerebral inoculation. These two viruses were chosen as models because of the ease with which they could be titrated, and because they seem to be extreme members of the virus spectrum. A few additional experiments with vaccinia, encephalomyocarditis (E.M.C.), Coxsackie, and poliomyelitis type III viruses are reported. The preliminary results with these viruses have confirmed our belief that the method to be described is of general application.

METHODS

Virus antigens

Freshly harvested material was used except where mentioned. Antigens obtained from very different sources were deliberately selected and no preliminary clarification was done apart from centrifugation at 2000 *g* for 10 min.

PR8 elementary bodies. Ten-day embryonated hen's eggs were inoculated intra-allantoically with the PR8 strain of influenza A virus. The seed virus was infected allantoic fluid stored at -70° and used at a dilution of 10^{-2} . The eggs were incubated at 37° for 48 hr. and chilled. The allantoic fluid containing the virus was removed and clarified by centrifugation; the haemagglutination titre was usually about 10,000 units/ml.

PR8 soluble antigen. Infected chorio-allantoic membranes from the same eggs were ground in a mortar with glass powder; a 20% (w/v) suspension was made in 0.15 M-NaCl, or later, in distilled water. The gross fragments of membrane and glass powder were removed by centrifugation.

Semliki forest virus. Three-week-old mice were inoculated intracerebrally with seed virus at a concentration sufficient to cause death in 2-3 days. Two

days later, when the mice were obviously sick, they were killed and the brains removed; a 20% (w/v) suspension was prepared, either in 0.15 M-NaCl or in distilled water. The suspension usually had an infectivity titre of about 10^8 LD₅₀/ml. when titrated by inoculating groups of mice intracerebrally.

Other viruses. Some preliminary experiments were made with viruses obtained from the following sources:

Vaccinia virus: from skin scrapings of infected rabbits.

E.M.C. virus: supernatant fluid from Krebs ascites cells infected in tissue culture; stored at -70° until used.

Coxsackie virus: a 20% (w/v) suspension of infected suckling mouse carcasses; stored at -70° until used.

Poliomyelitis type III virus: supernatant fluid from infected monkey kidney tissue culture, prepared by Glaxo Laboratories Ltd., and kindly supplied by Dr G. Boissard.

Virus antisera

Antisera specific for the PR8 and the Barratt strains of influenza A virus and for Semliki forest virus were prepared in guinea-pigs by infecting them with 0.5 ml. virus suspension intranasally or by intrapulmonary injection; the guinea-pigs were bled 2 weeks later. The serum was sterilized by filtration through a Millipore filter and stored at 4° . Monkey antiserum prepared against type III poliomyelitis virus was kindly provided by Dr Boissard.

Complement-fixation tests

Fresh guinea-pig serum stored at -70° was used as a source of complement; the technique of complement fixation was the plate method described by Fulton & Dumbell (1949) and Fulton (1958). For the titration of PR8 elementary bodies, a dilution of PR8 guinea-pig antiserum was selected which did not react with the PR8 soluble antigen. PR8 soluble antigen was titrated with a dilution of Barratt antiserum which reacted with PR8 soluble antigen but not with PR8 elementary bodies (Fulton & Isaacs, 1953).

Semliki forest virus guinea-pig antiserum was titrated directly with infected brain suspension; the highest dilution of antiserum which fixed complement maximally was selected for use.

Antigen fractions were compared by drawing antiserum contours; the titration end-point was taken as the highest dilution of antigen which fixed one unit of complement (that is, as the point where the antiserum contour cut the base line chosen as one unit of complement fixed).

Haemagglutination tests

Influenza virus elementary bodies were also assayed by haemagglutination. Fowl blood was stored in Alsever's solution at 4° for at most 7 days. The red cells were washed in 0.15 M-NaCl, diluted to approximately 0.5% (v/v), and accurately standardized by matching against a grey glass standard in a photometer. Twofold serial dilutions of virus in 0.15 M-NaCl were made on Perspex trays with a pipette delivering 0.12 ml., mixed with an equal volume of standardized red cells, and the patterns read as soon as the cells had settled.

Infectivity tests

Influenza virus. Infectivity was titrated by incubating virus dilutions with pieces of chorio-allantoic membrane and assaying for production of haemagglutinin according to the method described by Fulton & Armitage (1951).

Semliki forest virus. The virus was titrated by intracerebral inoculation of dilutions of the virus into groups of 5 mice.

Chromatographic technique

Calcium phosphate. The brushite form of calcium phosphate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) was prepared by the method of Tiselius *et al.* (1956). Into a flask fitted with a mechanical stirrer, equal volumes of 0.5 M- CaCl_2 and of 0.5 M- Na_2HPO_4 were run from two separating funnels, each at the same rate (about 120 drops/min.). The precipitate was allowed to settle and washed four times, by decantation, with distilled water. It was stored as a suspension in 0.001 M-phosphate buffer (c. pH 6.8), under which conditions it appears to be quite stable.

Preparation of columns. These were contained in glass tubes, into which it was convenient but not essential to incorporate a sintered glass plate to support the adsorbent, and with a tap at the bottom; the volume of dead space between sintered plate and tap should be kept as small as possible.

Glass tubes with diameters ranging from 5 to 40 mm. were used to accommodate columns containing from 1 to 200 ml. packed adsorbent. The length of the packed column was usually within the range of 2 to 5 times its diameter.

The success of the method depends to a large extent on the uniform packing of the columns which should be free from any cracks, channels or air bubbles. The packing was performed by first degassing the calcium phosphate suspension under vacuum and then pouring it into the tube, to which a little water had already been added. The tap was opened and the tube was tapped sharply to facilitate packing as the precipitate settled. Several volumes of water were then run through the column under slight positive pressure applied to the top of the column (not more than 60 mm. Hg). At no stage must the liquid level be allowed to fall below the top of the column. To avoid disturbing the surface when adding materials to the column, a small glass or polythene float was used as recommended by Partridge & Westall (1949).

We have found it possible to employ much higher flow rates than are recommended by other workers, without impairing the resolving power. Rates of the order of 0.5–1.0 ml./min./cm.² cross-sectional area were commonly used. Columns often run at this rate under atmospheric pressure; when necessary additional positive pressure may be applied, though it is important not to exceed the pressure which was initially used in packing the columns.

Before a column was used, its retention volume (volume of liquid held in the column) was determined by passing down a narrow band of a coloured indicator which was not adsorbed; an aqueous solution of bromocresol purple was used for this. By following the progress of the band, a useful check on the uniformity of packing of the column is obtained.

Operation of columns. As shown by Tiselius, calcium phosphate adsorbs

many large molecules but not small ones. Elution can be effected by increasing concentrations of phosphate buffer at a constant pH value, the *R* values changing rapidly from 0 to 1 over a very narrow range of phosphate concentration.

The crude source material was run through the column to adsorb virus or antigen. Elution then began with phosphate buffer, starting with dilute and proceeding to more concentrated solutions. Appropriate fractions were collected after first rejecting a volume corresponding to the retention volume of the column. By adding known volumes of buffer to the column, the point at which boundaries would appear in the effluent could be calculated; the location of these boundaries was often indicated visually by swirling effects where the concentration of buffer changed. Stock 1.0 M-phosphate buffer consisted of 0.66 M- Na_2HPO_4 and 0.33 M- KH_2PO_4 at pH 6.8. More dilute buffers were prepared from this; the pH values of these dilutions varied between 6.8 and 7.2 according to dilution.

Dialysis. Samples with a phosphate concentration greater than 0.2 M were dialysed against either water or 0.15 M-NaCl. This was necessary before they could be tested in a complement-fixation reaction, because high phosphate concentrations were anticomplementary. Dialysis was carried out in cellophan tubing, closed at one end by tying; the open end was attached to glass tubing to facilitate handling of infective material which could then be introduced or removed with a Pasteur pipette.

Protein estimation

Protein was estimated colorimetrically by the method of Lowry *et al.* (1951). The limit of sensitivity of the method is about 10 μg . protein/ml. In some experiments the figures obtained by this method were compared with protein-N figures on trichloroacetic acid precipitates, estimated by a modification of Johnson's method (1941); good agreement was obtained.

RESULTS

Purification and concentration of influenza virus elementary bodies

The results obtained in a typical experiment in which allantoic fluid infected with influenza virus PR8 was applied to a column and eluted with stepwise increasing concentrations of phosphate, are illustrated in Fig. 1. Concentrations of phosphate up to 0.2 M removed 90–95 % of the total protein but little of the haemagglutinin (HA) activity; most HA activity was eluted at 0.4 M, with a little more at 0.5 M. The routine method of purification finally adopted was to run the allantoic fluid through a column whose size was roughly equivalent to the volume of allantoic fluid used. (Larger volumes overload the columns in terms of protein though not in terms of HA activity.) The column was first washed with 0.2 M-phosphate, after which active material was eluted with 0.5 M-phosphate (see Table 1). Recoveries of 50–80 % were obtained. Material was obtained in this way with a purity up to 10^6 HA units/mg.

protein, representing a 30- to 100-fold purification as compared with the initial allantoic fluid.

Table 1. *Purification and concentration of influenza virus PR8 elementary bodies by using two columns*

Source material: 160 ml. allantoic fluid from chick embryos infected with influenza virus PR8. [The first three 0.5 M fractions from column 1 were pooled and diluted to 150 ml. (phosphate concentration 0.2 M) and applied to a second column.]

Phosphate concn. in eluent (M)	Vol. (ml.)	HA activity			Protein		HA units/mg. protein	Relative purifi- cation	Relative concen- tration
		Units /ml. $\times 10^{-3}$	Total units $\times 10^{-4}$	%	mg.	%			
		First column: retention vol. 170 ml.							
Starting material	160	24	384	100	243	100	15,800	1	1
0 (filtrate)	160	0.8	< 13	< 3.4	180	74			
0.2	100	2.26	22.6	5.9	68.5	28			
0.5	20	26.6	53.3	13.9	1.7	0.7	314,000	20	1.1
0.5	20	53.3	106.6	27.8	2.2	0.9	485,000	31	2.2
0.5	20	13.4	26.7	7.0	1.1	0.45	243,000	15	0.56
0.5	20	4.2	8.3	2.2	0.24	0.1			
			230.5	60.2	253.7	104.2			
Second column: retention vol. 18 ml.									
Starting material	150	12.4	186.6	100	5.0	100	373,000	24	0.52
0.2 (filtrate)	150	1.6	25	13.4	1.5	30			
0.2	10	26.6	26.6	14.2	0.5	10	532,000	34	1.1
0.5	3	666	200	> 100	1.3	26	1,530,000	96	27.6
0.5	3	333	100	53.4	1.0	20	1,000,000	63	13.8
0.5	3	26.6	8.0	4.3	0.3	6			
0.5	3	26.6	8.0	4.3	0.1	2			
			367.6	> 100	4.7	94			

By chromatography on a second smaller column a 10- to 30-fold concentration of HA activity was obtained, but there was little significant increase in purity (Table 1). The procedure was to dialyse or dilute the active fractions from the first column to decrease their phosphate content, adsorb them on the second column, and elute in small volumes of 0.5 M-phosphate.

High-titre concentrated material prepared in this way showed slight opalescence. On examination in the electron microscope all the visible particles appeared to be virus elementary bodies and all could be adsorbed on to chick red cells. A rough count on such material gave 8×10^{11} particles/ml. The infectivity of the virus was fully retained after chromatography on calcium phosphate.

Purification and concentration of influenza virus soluble antigen

Purification on one column is illustrated by the results shown in Table 2. The complement-fixation (CF) titres refer to the highest dilution of the fraction which will fix one unit of complement with a guinea-pig antiserum reacting with the influenza virus PR8 soluble antigen, but not with the PR8 ele-

mentary body. Most of the soluble antigen was recovered in a relatively broad band at 0.3 M and 0.4 M-phosphate.

The behaviour of the antigens in the complement-fixation test is illustrated in Fig. 2; by area, about 88% of the antigen initially present was recovered. The routine method finally adopted was similar to that described for virus elementary bodies, elution of inactive material with 0.2 M-phosphate followed by elution of activity with 0.5 M-phosphate.

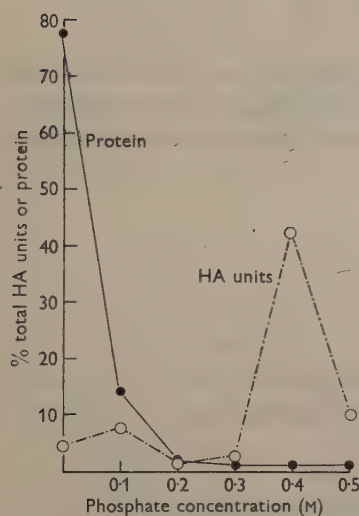


Fig. 1. Chromatography of influenza virus PR8 elementary bodies.

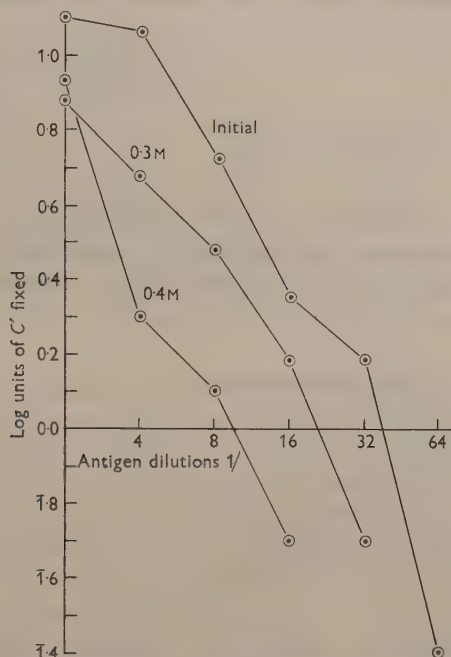


Fig. 2. Complement-fixing activity of influenza virus PR8 soluble antigen fractions after chromatography on one column.

Table 2. *Purification of influenza virus soluble antigen*

Source material: 30 ml. of a 20% (w/v) suspension in water of chick chorio-allantoic membranes infected with influenza virus PR8.

Column: retention vol. 27 ml.

Phosphate concn. in eluent (M)	Vol. (ml.)	CF titre (see text)	Protein		CF units/mg. protein	Relative purification
			mg.	%		
Starting material	30	36	150	100	7.2	1
0 (colourless filtrate)	25	—	21	14	—	—
0 (coloured filtrate)	5	<1	40	27	—	—
0.1 (coloured)	20	<1	102	68	—	—
0.2	20	<1	14	9	—	—
0.3	20	20	4	2.7	100	14
0.4	20	10	1.2	0.8	165	23
0.5	20	2	1.1	0.8	—	—
			183.3	122.3		

Purification and concentration on two columns is illustrated by the following experiment. A 20% (w/v) suspension in water (80 ml.) of chick chorio-allantoic membranes infected with influenza virus PR8 was purified on one column as above. The initial CF titre of the suspension was 20; elution with 30 ml. 0.5 M-phosphate gave a fraction with a CF titre of 40 (80% recovery). This fraction was diluted five-fold and run through a second column (retention vol. 10 ml.). The column was washed with 10 ml. 0.1 M-phosphate and the antigen eluted with 25 ml. 0.5 M-phosphate which was collected in three fractions:

0.5 M-phosphate fractions	Vol. (ml.)	CF titre
1	5	128
2	5	128
3	15	20

Fractions 1 and 2 contained most of the soluble antigen; when these two fractions were combined a concentration of 6.4-fold was achieved when a concentration of eightfold was expected. (Recovery on second column 100%; over-all recovery 80%.)

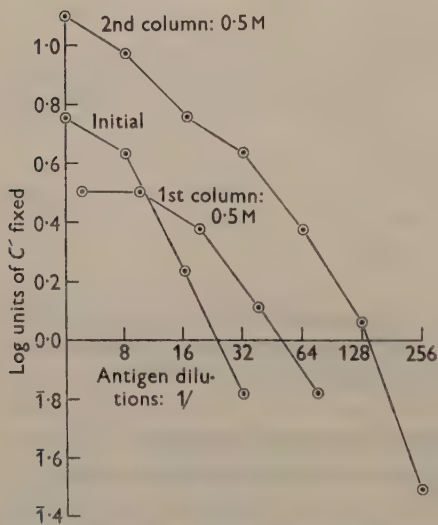


Fig. 3. Complement-fixing activity of influenza virus PR8 soluble antigen fractions after chromatography on two columns.

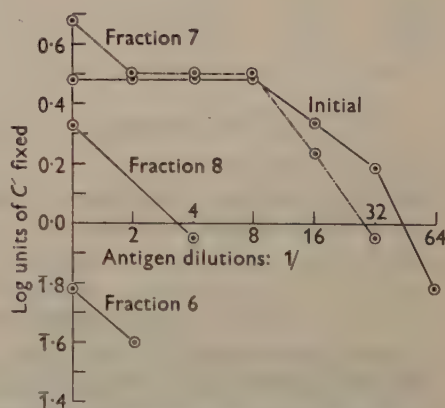


Fig. 4. Complement-fixing activity of Semliki forest virus after chromatography on one column.

The behaviour of the antigens in the complement-fixation test is illustrated in Fig. 3. There is a limit to the concentration of the soluble antigen that can be achieved by running through columns of progressively smaller capacity, as shown by failure to achieve the expected concentration; this limitation is probably due to the heterogeneity of the antigen which is also, presumably, the reason why it runs on the column in rather a broad band.

Purification of Semliki forest virus

The same general method was successful with Semliki forest virus. The results of a typical experiment are shown in Table 3. Most of the complement-fixing antigen was recovered in the fifth fraction (0.5 M) which contained only about 5% of the initial protein.

Table 3. *Purification of Semliki forest virus*

Source material: 40 ml. of a 20% (w/v) infected mouse brain suspension in water.
Column: retention vol. 40 ml.

Phosphate concn. in eluent (M)	Vol. (ml.)	CF titre (see text)	Protein		CF units/mg. protein	Relative purification
			mg.	%		
Starting material	40	43	124	100	13.9	1
0 (filtrate)	40	0	Not tested		—	—
0.1	100	0	32.3	26	—	—
0.2	80	0	8.8	7.1	—	—
0.5	20	Trace	2.2	1.8	—	—
0.5	20	25	6.2	5.0	81	5.8
0.5	20	4	2.2	1.8	36	2.6

The behaviour of the antigens in the complement-fixation test is illustrated in Fig. 4; by area, allowing for the expected twofold concentration, about 70% of the antigen initially present has been recovered. The antigen is principally the virus elementary body though there is, in addition, a very small amount of soluble antigen. The purified virus is still infective; no loss of titre could be detected by intracerebral titration in mice.

Purification of some other viruses

A few preliminary tests were made with other viruses: the results confirm our belief that the method is of general application, although the molarity of phosphate which elutes seems to differ according to the nature of the virus antigen. For example, a crude suspension of vaccinia virus obtained from skin scrapings of an infected rabbit was adsorbed on a column of calcium phosphate. The column was washed with 0.1 M-phosphate and the complement-fixing antigen was eluted with 0.5 M-phosphate; this fraction contained large numbers of virus elementary bodies.

Again, E.M.C. virus contained in the supernatant fluid from Krebs ascites cells infected in tissue culture, was adsorbed on a column, washed with 0.1 M-phosphate and eluted with 0.2 M-phosphate. The virus in this fraction was still infective. Coxsackie virus contained in a 20% (w/v) suspension of infected suckling mouse carcasses could be adsorbed and eluted in a similar manner, and was still infective. Finally, poliomyelitis virus type III contained in the supernatant fluid from infected monkey kidney tissue culture was adsorbed on a column and washed with 0.1 M-phosphate. Subsequent elution with 0.5 M-phosphate removed the virus quantitatively as judged by complement fixation.

DISCUSSION

Most of the purification of the virus antigens by chromatography on calcium phosphate is achieved on the first column. We have shown that in at least one instance (the influenza virus elementary body), where the adsorption band is narrow, it is possible to obtain a concentration of the order of 30-fold by the use of a sequence of progressively smaller columns. In general, however, it seems probable that a heterogeneous antigen which tends to run through the column in rather a broad band could, after purification on the first column, be more conveniently concentrated by other means. However, when fractionation of a heterogeneous antigen is required, rather than its over-all concentration, a sequence of columns is likely to be very effective.

The purity of influenza virus elementary bodies after chromatography on calcium phosphate, 10^6 HA units/mg. protein, compares favourably with that reported by other workers who used other methods of purification, even allowing for the uncertainty inherent in haemagglutination tests. Miller & Schlesinger (1955) purified the Melbourne strain of influenza virus by chromatography on aluminium phosphate and reported a purity of 500,000 HA units/mg. N; Ada & Perry (1956) who purified influenza virus PR8 by red cell adsorption and centrifugation, and Burke, Isaacs & Walker (1957), who purified three strains by chromatography on aluminium phosphate and centrifugation, both reported about 100,000 HA units/mg. dry weight.

Columns of calcium phosphate appear to offer several advantages over columns of aluminium phosphate, provided a suitable crystalline form of calcium phosphate be used. They are easier to prepare and give good flow rates without the necessity of mixing with an inert substance. Other advantages are that crude material can be used without preliminary purification and all operations can be carried out at neutral pH values. The use of a reproducible crystalline form of calcium phosphate packed in columns possesses obvious advantages compared with the technique of adsorption on a precipitate formed by adding calcium chloride to a phosphate-containing solution. Stanley (1945) reported unfavourably on the latter technique for the purification of influenza virus PR8; one factor which must have influenced his results was the variation from one experiment to another of the concentration of phosphate remaining in solution after formation of the precipitate, so that optimum conditions for adsorption were seldom achieved.

We should like to thank Dr A. Isaacs and Dr R. C. Valentine for their kindness in examining the purified influenza virus elementary bodies with the electron microscope, and Mr D. Garwes for his skilful technical assistance. One of us (J.T.) was in receipt of a personal grant from the Medical Research Council.

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Since this paper was submitted for publication a short paper on the purification of viruses and rickettsiae on columns of modified cellulose by Hoyer, B. H., Bolton, E. T., Ormsbee, R. A., Le Bouvier, G., Ritter, D. B. & Larson, C. L. (*Science*, 1958, **127**, 859) has appeared, in which they use a method very similar to the one we have described. The preparation of modified cellulose adsorbents is, however, more difficult than the preparation of calcium phosphate, and on the basis of these results it is not yet possible to say whether modified cellulose will prove as useful in virus purification as calcium phosphate. We have not yet been able to make a direct comparison of the two adsorbents.

Some Studies on the Occurrence and Properties of a Large Gram-Negative Coccus from the Rumen

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SUMMARY: A large Gram-negative coccus was isolated in a lactate-containing medium from the rumen of young calves. This organism is anaerobic and ferments only lactate, glucose, fructose, maltose, sucrose, mannitol and sorbitol. The coccus is found in large numbers (c. 10^9 /ml.) in the rumen contents of young calves, but is present only in small numbers or is absent from the rumens of older cattle and sheep. An attempt made to establish the organism in large numbers in a sheep rumen was unsuccessful. The possibility of the coccus being a normal inhabitant of the throat of adult animals was also examined. Five serological types were obtained. The distribution of these types in the same animal over a period, and in different animals, appears to be random. The distribution of a small lactate-fermenting coccus (*Veillonella gazogenes*) is also described.

During the last few years a large number of bacteria have been isolated from the rumens of cattle and sheep, and some of these have been the subjects of extensive study. The present paper deals with the properties and occurrence of a large Gram-negative coccus which has been found to be common in the rumens of young animals, but less so in older animals. The organism was first isolated by chance in media used for cultivating lactobacilli and when its properties became known it was sought specifically in rumen contents. A preliminary communication about this organism has already been made (Hobson, Mann & Oxford, 1958).

METHODS

Media. Tomato extract agar was prepared by the method of Rogosa *et al.* (1953). The semi-solid medium contained Bacto casitone, 6 g.; Bacto yeast extract, 2 g.; L-cystine, 0.3 g.; thioglycollic (mercaptoacetic) acid, 0.12 ml.; NaCl, 2 g.; agar, 0.3 g.; sodium lactate solution (70%, w/w), 5.7 ml.; resazurin, 0.4 ml. of 0.1% (w/v) solution; distilled water, 400 ml. Sterilized by autoclaving at 121° for 15 min. The pH value of the medium was 6.9-7.0. Increasing the agar concentration to 2% gave solid thioglycollate medium. Johns's medium was prepared as described by Johns (1951). This was made into Chocolate Johns's Agar (CJA) medium by addition of 5% (v/v) heated blood. Elsdén's media A and B were prepared by the method of Elsdén *et al.* (1956). The semi-solid medium (9 ml.) was incubated in tubes (6 in. \times $\frac{5}{8}$ in.) in air, other media under 5% (v/v) CO₂+95% (v/v) H₂, and all at 38°. Sodium lactate solution was a 70% (w/w) solution (Hopkin and Williams Ltd.,

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Chadwell Heath) and the sugars used were the standard grades supplied by L. Light, Colnbrookdale, T. Kerfoot, Vale of Bardsley, or British Drug Houses Ltd. All media mentioned later in the text contained sodium lactate and no carbohydrate unless described otherwise.

Isolation of the organism. The final isolation method was as follows. Samples were taken from animals fitted with permanent rumen cannulae or by stomach tube, and when necessary lightly centrifuged to remove the larger debris. Serial tenfold dilutions were then made in the semi-solid medium and these were incubated for 2 days. The tubes were examined for growth of the large coccus and subcultures were made, generally from the highest dilutions in which growth was occurring, on to CJA medium or on solid thioglycollate medium. Further subcultures were made into Robertson's cooked meat medium and back on to CJA or solid thioglycollate media until a pure culture was obtained.

Preservation of the organism. Stock cultures were kept in Robertson's cooked meat media subcultured every 5–7 days. Representative cultures of the different serological types, grown in Johns's medium, were freeze dried from glucose + serum suspension after the organisms had been washed once.

Biochemical reactions. Fermentation reactions were determined by using the semi-solid medium adjusted to pH 7.0–7.2, containing 1 % (w/v) substrate and 0.1 % (w/w) of a 1.6 % (w/v) ethanolic solution of bromocresol purple. Tests for formation of H_2S and indole were made with the semi-solid medium, the oxidase and catalase tests on CJA medium, and gelatin liquefaction in the semi-solid medium with the agar replaced by 12 % (w/v) gelatin.

Determination of fermentation products. Total volatile fatty acids were determined by steam distillation in the apparatus of Markham (1942) and titration of the distillate with 0.05 N-NaOH in a stream of CO_2 -free air. For qualitative determination of the acids present the distillate was evaporated to a suitable volume and analysed chromatographically on filter paper by the method of Elsdon & Lewis (1953). The acids were analysed quantitatively, after a second distillation with mercuric salts (Friedemann, 1938) by evaporating the alkaline extract to dryness, and extracting with a chloroform + butanol mixture (Elsdon, 1946). A portion of this solution was titrated with ethanolic NaOH to determine the total acid present, and further samples were analysed on silica columns as described by Moyle, Baldwin & Scarisbrick (1948). Gases were determined by cultivating the organism in a completely filled flask connected by a capillary to a mercury filled gas burette. CO_2 in the gas evolved was absorbed in barium hydroxide solution and the residual gas, which was explosive, was assumed to be hydrogen.

Serological methods. Preparations of antigens were made from organisms from a 48 hr. culture in Johns's medium; they were centrifuged down, washed three times with saline, suspended in saline, and heated at 60° for 1 hr. These suspensions, with the addition of 0.5 % (w/v) phenol, were kept at 2°.

The method given by Browning & Mackie (1949) for preparation of antisera to meningococci was used for preparing antisera. Usually two series of injections produced a good antiserum. Doubling dilutions of the antisera were

tested for agglutination reactions in tubes. Rapid serological tests were made with undiluted antisera for slide reactions. Antisera which cross-reacted in agglutination tests were absorbed by a heavy suspension of the appropriate organism until no further reaction occurred.

Fluorescent antisera were prepared and liver-absorbed as described by Hobson & Mann (1957). Tests were made by methods also described in this paper.

Extracts of organisms were made in 0.15 % (w/v) NaOH by shaking a thick suspension with ballotini beads for 45 min. at room temperature. The disintegrated cells were removed by filtration through a Berkefeld filter and the extract neutralized with dilute HCl when necessary.

Precipitin tests were carried out by layering the extract on to the antiserum in a small tube; an interfacial precipitate was looked for after incubation at 37° for 30 min.

Growth at different pH values. This was tested by using the semi-solid medium buffered with citric acid + KH_2PO_4 mixtures. The pH values of the cultures and uninoculated controls incubated together were measured with a Pye pH meter.

Tests for formation of iodine-staining polysaccharide. The colonies growing on CJA medium containing either lactate, glucose or maltose were flooded with iodine at intervals, and the reactions of colonies and individual organisms noted.

Identification of the small Gram-negative coccus sometimes cultured with the large coccus. The fermentation reactions of the small coccus were determined by using a semi-solid medium; other tests were as for the large coccus. The small and large cocci could be distinguished on CJA medium, the large coccus having a greenish colony, and the small coccus a greyish-white colony.

RESULTS

The first isolations were made on tomato extract agar which gave growth corresponding to 10^4 organisms/ml. rumen contents from a 5-day-old calf. When it was found that the organism metabolized lactate, isolation in a lactate-containing medium was attempted and it was found that the procedure described above was satisfactory. In a later experiment samples from calves l and m at 3 weeks old (Table 4) were diluted and cultured in Elsden's media A and B. In medium A, containing starch, the coccus grew only in dilutions corresponding to numbers of 100/ml. rumen contents, and small Gram-positive cocci, probably *Streptococcus bovis*, grew in dilutions corresponding to 10^9 /ml. In medium B, containing lactate, the large coccus grew in dilutions corresponding to 10^9 or 10^{10} /ml. which compared well with the results of the normal isolation procedure. Elsden *et al.* (1956) found that a lactate-containing medium inoculated with rumen contents produced a dense growth of *Veillonella gazogenes* which completely overgrew the large coccus and made it impossible to isolate. Although we generally obtained a mixed growth of organisms in the lactate-containing medium, only in one or two cases were small Gram-

negative cocci present in numbers great enough to prevent the isolation of the large coccus. A number of isolations were made of small cocci which from their morphology, ability to ferment only lactate, pyruvate, malate and fumarate to give acid and gas, together with the fact that they were anaerobic, were identified as *Veillonella gazogenes* (Johns, 1951).

The large coccal organism is usually Gram-negative, but some organisms have a more purplish colour than the majority and occasionally a coccus is found which seems to be Gram-positive. The morphology of the coccus is shown in Pl. 1, fig. 1-4. The diameter of cocci in fixed preparations is usually about $1.5\ \mu$, but this varies and occasional large forms are found. These large forms, which often become granular, become more numerous as the cultures become older until they predominate. Old cultures autolyse rapidly with the formation of granular masses of debris, and the autolysis seems to be most rapid between pH 5 and 7. The cocci usually occur in pairs, and sometimes singly, but chains are rare, except in young cultures. It has not been found possible to distinguish a capsule by normal negative staining methods, but fluorescent antisera seem to demonstrate a small capsule or slime layer varying in thickness from coccus to coccus. The organism generally occurs in the rumen as isolated diplococci and in small groups, but one large group reacting with fluorescent antiserum is shown in Pl. 1, fig. 5, 6.

There are some differences in colony type amongst the organisms isolated. Serological type 2 has colonies 1-1.5 mm. diameter, with raised centres, greenish yellow in colour, and with flat greyish peripheries with an uneven edge and smooth surface. Smaller colonies do not show the flat periphery. The other types have a similar colony, but the centre is not as raised or pigmented and the flat periphery is more extended. Deposits of organisms from liquid cultures also have a greenish colour.

The organism is an obligate anaerobe and in liquid or on solid media is grown under 5% (v/v) CO_2 + 95% (v/v) H_2 , but it does not need this concentration of CO_2 and will grow under H_2 alone or in the depths of the semi-solid medium. In liquid cultures it needs a large inoculum to start growth, but it does not require a rumen liquid supplement as do some rumen bacteria.

The fermentation reactions are shown in Table 1. Twenty-one isolations, representing serological types 1-3, were tested for fermentation reactions. With the exception of sorbitol, which was fermented by types 1 and 3, but not by type 2, no variation in reactions was found. H_2S is formed, but not indole, catalase or oxidase. Gelatin is not liquefied.

When grown on lactate the pH value initially decreases, but then increases to 7 or slightly above; CO_2 and H_2 and volatile fatty acids are formed. In one experiment the acids formed (as m-equiv % of total m-equiv of acid formed) were: valeric 12.6, butyric 13.7, propionic 38.2, acetic 35.5. In the same basal medium growth on glucose was slower, the final pH value being 4.9; CO_2 and H_2 were again formed together with fatty acids. In this case little or no propionic acid, but some caproic acid, was formed. The proportions of the different acids differed in different experiments, and at different times in the same experiment, but the same acids were found each time. In one experiment

Table 1. *Fermentation reactions of the large Gram-negative coccus*

Semi-solid medium as described in text. Substrates 1 % (w/v). Incubation 14 days at 37°.

<i>Substrate</i>	<i>Reaction</i>	<i>Substrate</i>	<i>Reaction</i>
Fructose	+	Aesculin	—
Glucose	+	Salicin	—
Galactose	—	Mannitol	+
Xylose	—	Sorbitol	+
Arabinose	—	Dulcitol	—
Rhamnose	—	Inositol	—
Maltose	+	Glycerol	—
Sucrose	+	Adonitol	—
Trehalose	—	Sod. lactate	+
Lactose	—	Sod. succinate	—
Raffinose	—	Sod. fumarate	—
Dextrin	—	Sod. formate	—
Starch	—	Sod. malate	—
Inulin	—	Sod. alanate	—

+, Acid plus gas; —, no action. Growth on sucrose was slower than on other sugars; fermentation of sorbitol varied with the serological type (see text).

the acids formed were: caproic 5.9 %, valeric 6.7 %, butyric 52.1 %, propionic 1.3 %, acetic 33.9 %, respectively, of the total volatile fatty acids recovered after distillation with mercuric salts. In cultures grown on glucose there was always a volatile acid present (about 6–10 % of the total) which was destroyed during this distillation and which was probably formic acid. The only carbohydrate to be detected chromatographically in the culture filtrate after growth on glucose was glucose; no soluble oligosaccharides appeared to be formed.

Growth occurred over the range of pH 4.8–8.1. The preliminary tests for formation of iodine-staining polysaccharide in the cocci showed that they became filled with material giving a dark brown, often granular, glycogen-like stain with iodine after about 40 hr., and that this was lacking after 24 hr. incubation; but these times varied with the rate of growth. The colonies became dark brown staining with iodine at the same time, and this could easily be distinguished from the normal light brown colour of the colonies with iodine.

The results of experiments in which counts of the numbers of cocci present in the rumen contents of a number of calves and heifers were made are shown in Table 2. The fluorescent antiserum technique was used to test for the presence of cocci in these and other rumen contents as an additional check on the isolations and also to try to determine whether the large cocci visible in some samples, but which would apparently not grow on the media used, were in fact the lactate-utilizing organism. The calves were getting a variety of diets at the times of sampling, from a completely milk diet to a concentrate and hay diet for the oldest ones. The environments of the animals also differed, and the samples were taken at various times over some two years. Table 3 shows the distribution of these cocci in four adult sheep sampled over a period. During this time the environments and diets were constant. Sheep 21 was getting a diet of hay, potato starch and concentrates, sheep 86 and 879 concentrates and hay, and sheep 9 hay and grass cubes. Two calves, l and m,

Table 2. *Distribution of large Gram-negative cocci in cattle*

Animal	Age at time of sampling	Isolation at/ml.	Found with fluorescent antisera types 1 and 2
Calf			
a	20 weeks	10 ¹ , 10 ²	—
b	20 weeks	0	—
c	8 weeks	10 ⁹	.
d	6 weeks	10 ¹⁰	++
e	8 weeks	10 ⁹	.
f	5 weeks	10 ⁴	—
g	7 weeks	10 ¹⁰	++
h	7 weeks	10 ⁸	.
i	9 weeks	10 ⁸	.
j	3 weeks	10 ¹⁰	±*
k	6 weeks	.	++
Heifers†			
6	15 months	0	± or —
Heifer			
1	15 months	0	+
2‡		0 or 10 ⁵ §	± or — or ++§
3		0	++
Heifer			
1a	15 months	0	—
2a		0 or 10 ⁵ §	— or ±§
3a		0	±

Calves of the same age were not necessarily sampled at the same times. The tests cover a period of some 2 years.

* Isolated cocci were different serological type to antisera used for testing.

† Grass diet.

‡ Silage diet. Samples at intervals over 1 month.

§ Corresponding samples.

|| Roots diet.

—, No large cocci reacting; ±, few; +, ++, increasing numbers.

., No test.

were sampled for these cocci at intervals from 3 weeks old. The diets of both were the same, the calves being weaned 1 to 2 days before the first sample and thereafter a concentrate mixture, made up from groundnut and molasses meals, flaked maize, bruised oats, minerals and vitamins, and a little hay were given. The cocci were present in both calves for the whole time of sampling (Table 4). The isolation of these cocci from kids was not attempted but observations of stained films of kid rumen contents showed very few large Gram-negative cocci.

Serology

An antiserum to one of the original isolates from a calf was prepared and other isolates were tested against this antiserum. One of the isolates did not react with this antiserum in high dilution and this was used for preparing a second antiserum. Subsequent isolates of large cocci on plate cultures, usually derived from the highest dilution primary cultures which showed growth, were rapidly tested by the slide-agglutination technique. Only the representatives of new types were then kept for more extensive tests. Tests of some 250 isolates

Table 3. *Distribution of large Gram-negative cocci in sheep*

Sheep	Test no.	Found with	
		Isolation at/ml.	fluorescent antisera types 1 and 2
No. 21	1	.	±
	2, 3, 4	.	—
	5	0	—
	6	10 ⁴	.
	7	10 ⁵	.
	8	10 ⁴	.
No. 879	1	.	—
	2	10 ¹	.
	3	10 ⁴	.
	4	10 ⁶	.
No. 9	1	0	±
	2	0	.
	3	0	.
	4	0	.
No. 86	1	0	.

Symbols as Table 2.

Sheep 21, tests 1–5 over 1 year, 6, 7, 8 over 1 month.

Sheep 879, tests 1 and 2, 5 months interval, tests 2–4 over 1 month.

Sheep 9, tests 1 and 2, 4 months interval, tests 2–4 over 1 month.

Table 4. *Distribution of different serological types of the Gram-negative coccus in two calves*

Age of calf (weeks)	Calf 1				Calf m				Total count for each calf/ml.
	% of isolations belonging to coccus type				% of isolations belonging to coccus type				
	1	2	3	4	1	2	3	4	
3	10 ^{10*}
4	0	100	0	0	100	0	0	0	10 ¹⁰
	—	++			—	±			
6	0	100	0	0	96	4	0	0	10 ¹⁰
	—	+			—	—			
8	5	16	79	0	100	0	0	0	10 ⁸
	—	+			+	±			
10	0	23	62	15	50	0	50	0	10 ⁹
	—	±			—	—			
12	0	0	80	20	0	0	100	0	10 ⁹
	—	—			±	—			
14	8	0	0	92	93	0	7	0	10 ⁸
	—	—			+	±			

—, +, Fluorescent antisera tests, symbols as Table 2.

* Not tested serologically.

from calves yielded three distinct serological types, with a few organisms belonging to what appeared to be a fourth type. A fifth type was isolated from sheep 879. The relationships between the types are shown in Table 5. There was some cross-reaction between the organisms and antisera types 1 and 2 which was discernible with the fluorescent antisera, and was apparent with slightly diluted (e.g. 1/80; titre with homologous organism 1/5,120) antisera in

tube agglutination tests. This cross-reaction was removed by absorbing the antisera with the appropriate organisms. The different serological types have been stable on sub-cultivation for about 2 years, with the exception of type 4 which rapidly lost the antigen which reacts with type 2 antiserum. No cross-agglutination reaction of antisera types 1 or 2 with strains of *Neisseria catarrhalis*, *Veillonella gazogenes* or *Sarcina bakeri* (a rumen Gram-negative coccus) occurred. The dilute NaOH extract of any one serological type reacted in precipitin tests with all the antisera, suggesting the presence of a common group antigen. No group-reacting substance was extracted by the acid or formamide methods usually used for streptococci.

Table 5. *The relationships of the serological types of Gram-negative cocci as shown by agglutination tests*

Type	Antiserum		
	1*	2*	3
1	+	—	—
2	—	+	—
3	—	—	+
4	+	+	—
5	—	—	—

+, Reaction; —, no reaction.

* Antisera absorbed to remove cross-reaction between types 1 and 2.

Serological type 2 is the most common in the calf rumen, nearly all the isolations from the calves mentioned in Table 2 which were tested belonged to this type, with a few type 1. Types 3 and 4 only appeared in the later tests on calves 1 and 2. However, the results of detailed examination of the calf rumen isolations given in Table 4 show that the serological types fluctuate quite markedly and it is possible that more extended observations on the calves mentioned in Table 2 would have shown a wider variety of types. The discrepancy between the observations with fluorescent antisera and tests on isolated cocci from calf 2 at 4 and 6 weeks old cannot be explained. Samples from other calves from which no isolations were made have been tested with the fluorescent antisera and organisms of types 1 and 2 have been found, together with some morphologically similar cocci not reacting with either antiserum. The calf serological types 1 and 2 were found in sheep as well as a new type (5).

Attempted establishment of the organism in the rumen

In an attempt to establish the organism in large numbers in the rumen of a sheep a suspension of cocci of serological type 2, from a 48 hr. CJA culture, containing approximately 5×10^9 cocci, was injected through the cannula into the rumen of a sheep which showed only type 5 cocci. Viable counts of the cocci, serological tests on the isolated organisms, and tests of the rumen contents with type 2 fluorescent antiserum were made at intervals up to 7 days after the inoculation, but the added organisms did not grow. The sheep had

been selected because it had 10^4 or less type 5 cocci/ml. rumen contents, but on the day of inoculation this had gone up to 10^6 /ml.

Cultures were also made from throat swabs of sheep 21 at intervals. However, few large Gram-negative cocci were seen in films made from the swabs, and although some grew in culture, the main organisms cultured were *Veillonella gazogenes*.

DISCUSSION

From a comparison of their morphological and biochemical properties there is no doubt that the organism described here is that described by Elsdén *et al.* (1956) under the name LC, and who discussed the taxonomic position of the organism and its pathway of lactate fermentation. However, these authors although they obtained the organism from a number of sheep did so by an indirect method, using medium A as an enrichment culture and had no knowledge of the numbers present in sheep, or the distribution of the organism in other animals. Information about this, and about some of the serological properties of the organism, together with other properties not discussed by Elsdén *et al.* (1956), are given in this paper. The results obtained would seem to show that while the organism is one of the major ones present in the rumen of the young animal it is not present in sufficient numbers in the adult animal to be of great significance. Even the ingestion of a diet containing large amounts of lactic acid, such as the silage fed to the heifers (Table 2), did not lead to the growth of an appreciable number of the cocci. (Cultural examination of the silage itself showed no large cocci.) The reason for the diminution in numbers as the animal grows older is being investigated. Microscopic observations on some calves of about 3 weeks old suggested a correlation between numbers of Gram-negative cocci and numbers of large Gram-positive rods (presumably lactobacilli) but no cultural work to confirm this has been done. The sheep isolations, and the failure of the attempt to establish the organism in the rumen of a sheep, suggest that there is some mechanism which limits the total numbers in the adult animal to less than *c.* 10^6 cocci/ml. The latter experiment and the need, in general, for a large inoculum to start cultures growing make it somewhat difficult to understand how the organism becomes established in the rumen, and the variation in numbers suggests that it is not well adapted to conditions there. The possibility that the organism's natural habitat is the throat and that the organisms in the rumen come from there led to the attempted isolation of the coccus from throat swabs. The results obtained so far do not support this possibility, as the small number of organisms isolated could quite easily have come from particles of food lodged in the throat after regurgitation from the rumen. The fluctuations in serological types in the calf rumen (Table 4) are quite wide, but similar results have been found for changes in serological types of *Escherichia coli* in the human intestinal tract (Sears & Brownlee, 1952).

In animals from which the organism was not isolated large Gram-negative cocci (not reacting with the fluorescent antisera) were often seen, and it would appear that the coccus described here is only one of a number of morphologi-

cally similar organisms in the rumen. A large Gram-negative tetracoccus (*Sarcina bakeri*), which also occurs in diplococcal form, was isolated some years ago from the sheep rumen (Mann, Masson & Oxford, 1954), but this has no relationship to the coccus described here, and further tests where we have failed to isolate *S. bakeri*, or detect it serologically, suggest that this, as well, is only one of a number of Gram-negative diplo- and tetra-cocci present in the rumen.

The large Gram-negative coccus was nearly always accompanied, in the lower dilutions at least, in rumen samples incubated in a lactate-containing medium, by *Veillonella gazogenes*, and the numbers in which these occurred supported the observations of Johns (1951) and Gutierrez (1953) that *V. gazogenes* is generally present in numbers of about 10^4 /ml. sheep rumen contents. In the calves and heifers they were usually present in about the same number, although they were once found at 10^8 /ml. in a heifer. However, direct observation of sheep or cattle rumen contents shows the presence of very large numbers of small Gram-negative organisms which might be *V. gazogenes*, and this does not accord with the comparatively small numbers isolated.

The authors wish to thank Miss M. Garvock and Miss S. Bell for technical assistance, and a number of colleagues for providing rumen samples.

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EXPLANATION OF PLATE

Figs. 1-4 are phase contrast, $\times 704$, of wet mounts taken directly from cultures.

Fig. 1. Normal growth of the large Gram-negative coccus.

Fig. 2. Older culture showing larger forms, and also chain formation normally seen mainly in young cultures.

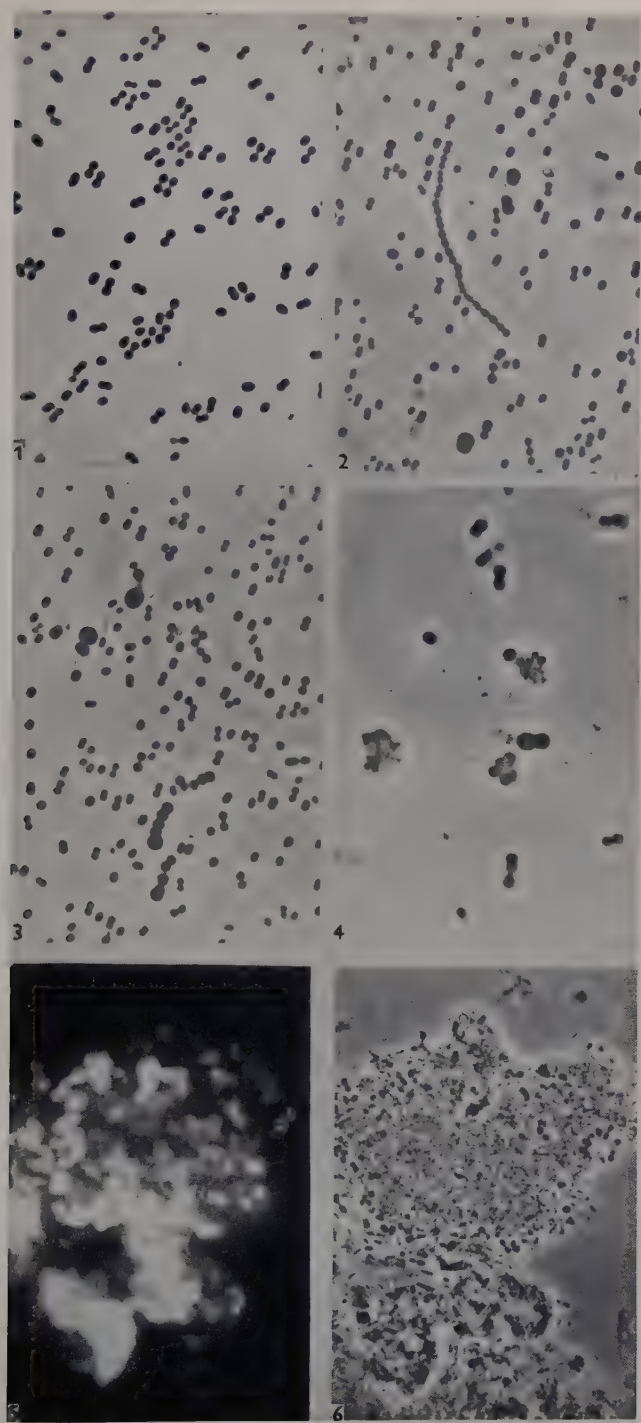
Fig. 3. Older culture showing many swollen forms.

Fig. 4. Final stages of a culture showing swollen and disintegrating cells.

Fig. 5. Coccus type 2, in the rumen contents of a 2 month old calf receiving a solid diet, reacting with fluorescent antisera. Background fluorescence blue-white. Ultra-violet, $\times 680$.

Fig. 6. Same field as fig. 5. White light phase contrast $\times 680$.

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P. N. HOBSON, S. O. MANN & A. E. OXFORD—A RUMEN GRAM-NEGATIVE COCCUS.
PLATE 1

(Facing p. 472)

MILLS, R. F. N. (1958). *J. gen. Microbiol.* **19**, 473-481.

The Effect of Infecting the Cells of the De-embryonated Egg with Influenza Virus on their Uptake of Glucose and Amino Acids

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SUMMARY: A method was found for infecting all the cells of the de-embryonated hen's egg with influenza B virus (strain Lee). Infected cells took up more glucose from the culture medium than did uninfected cells. Neither infected nor uninfected cells took up any of six amino acids, given singly. A mixture of thirteen amino acids was taken up well, and to the same extent, by infected and uninfected cells. When cysteine, histidine or methionine was omitted from this mixture, the remaining amino acids were taken up better by uninfected cells than by infected cells; when any other amino acid (arginine, glutamine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, tyrosine, valine) was omitted there was no significant difference in uptake by infected and uninfected cells. In this system, ethionine delayed multiplication of influenza virus, but β -phenylserine did not. These results are consistent with an increased requirement of the infected cell in this system for cysteine, histidine and methionine.

Amino acid analogues, e.g. methoxinine and ethionine (Ackermann, 1951*b*) and phenylserine (Dickinson & Thompson, 1957) have been found to inhibit virus growth in tissue culture in concentrations tolerated by the tissue; their activity was annulled by the corresponding natural amino acids. This suggested that virus-infected cells needed more of certain amino acids than did uninfected cells. In turn, knowledge of increased needs for amino acids by virus-infected cells might act as a guide to other amino acid analogues capable of inhibiting virus growth. The present work reports a search for such increased needs by measuring the uptake of amino acids by normal and virus-infected cells.

For this work it seemed advisable to use a uniform population of cells and a non-cytopathogenic virus. The cells of the de-embryonated hen's egg, which are said to be very uniform (Henle, 1953), and influenza B virus, strain Lee, were chosen. Lee virus has the added advantage that it gives rise to 'incomplete' virus less readily than do other strains of influenza virus (Fazekas de St Groth & Graham, 1954), so avoiding possible complications from this cause. To make any effect of infection as clear as possible it was necessary to infect all the exposed cells; it also seemed likely that decreasing the concentration of internal metabolites by starvation might make effects of infection on uptake clearer. In view of this, preliminary studies were made to find the effect of starvation on virus production and the conditions necessary for infecting all the cells. A study of the effect of infection on glucose uptake similar to those of Ackermann (1951*a*) and Bauer (1953), made as a preliminary to work on amino acid uptake, is also reported here.

METHODS

Viruses. Influenza B virus (strain Lee) was propagated in the allantoic sacs of 10-day eggs, harvested after 48 hr., and stored at -60° as a 1/100 dilution of infected allantoic fluid in Lab-Lemco broth. For experimental use, 0.1 ml. of a 1/10 dilution of stock virus was inoculated into the allantoic sacs of 9-day eggs; the allantoic fluid was harvested after 42 hr. incubation and used immediately.

Influenza A virus (strain PR 8) was used in some experiments on interference. It was propagated, stored and used in the same way as Influenza B virus.

Both strains were estimated by a modification of the method of Salk (1944). Serial twofold dilutions of 0.25 ml. infected fluid were made in normal saline in cups in a Perspex plate; 0.25 ml. of a 0.5% (v/v) suspension of washed fowl red blood cells was added; the mixture was shaken and allowed to settle for 1 hr. The end-point was taken as the last cup showing complete patterning, and the result expressed as the reciprocal of the final dilution of infected fluid.

Cells. The cells of the de-embryonated hen's egg (Bernkopf, 1949) were used. The eggs were 14–15 days old when de-embryonated.

De-embryonation. The method of Finter, Liu, Liebermann & Henle (1954) was used. Eggs were sealed with bottle-caps waxed in position, or more conveniently with the rubber liners of blood transfusion bottle caps (which stayed in place without waxing). The eggs were rotated at 5 rev./hr. during the experiments.

Media. After de-embryonation the eggs were washed out with physiological saline. For all other work a balanced salt solution (BSS) containing the salts of the medium of Hanks & Wallace (1949) was used. The basal medium was supplemented as required with amino acids and with glucose (0.5 mg./ml. for work on glucose uptake, 1 mg./ml. for work on amino acid uptake).

Glucose estimation. The method of Somogyi (1945*a*) was used, the fluids being deproteinized with barium hydroxide and zinc sulphate (Somogyi, 1945*b*) and the precipitate removed by centrifugation. The method was accurate to 2% at a glucose concentration of 0.5 mg./ml. It was sensitive to 5 μ g. glucose/ml.

Amino acid estimation. The colorimetric method of Smith & Agiza (1951) was used. More satisfactory results were obtained by omitting the sodium chloride, and by extracting the coloured material into 5 ml. *n*-butanol added from a pipette. The accuracy of the method was 5% for 10 μ g. α -amino-N/ml.; it was sensitive to 0.2 μ g. α -amino-N/ml.

In all estimations the glucose or amino acid in the original medium was used as a standard. For colorimetry a series of concentrations of amino acid in BSS was always prepared to test the linearity of the colour response: this linearity held to at least twice the concentration of amino acid used experimentally.

Plan of experiments

Apart from some preliminary experiments, described below, and tests of amino acid analogues for ability to decrease virus growth, the following general

plan was adopted. After the eggs had been de-embryonated and washed twice with saline, 10–15 ml. BSS without glucose was pipetted into each egg and the eggs were then rotated for 24 hr. After this preliminary starvation the fluids were decanted from the eggs. The eggs were then rinsed-out with 5 ml. BSS (which now, and in the rest of the experiment, contained glucose and was supplemented with amino acids as required), and filled with 10–15 ml. of the same fluid. After this, one of two treatments was used. For work on glucose uptake and some early work on amino acid uptake the eggs were rotated 24 hr., then the fluids removed for titration of residual substrate. The eggs were then rinsed-out, refilled with fresh medium, and infected with 0.1 ml. undiluted influenza B. The eggs were then rotated 24 hr., and the fluids removed and saved for haemagglutinin titration. The eggs were rinsed-out and refilled as before, rotated 24 hr., then drained and the fluids saved for estimation of haemagglutinin and residual substrate. Uninfected controls were run at the same time and also infected and uninfected eggs for which no amino acids had been added to the BSS + glucose solution. Uptake of glucose on the second and fourth days was measured and the change in uptake for each egg found. The effect of infection was obtained by comparing the changes in uptake for infected and uninfected groups of eggs.

This method resulted in a big leakage of ninhydrin-positive material into the medium towards the end of the experiment. To shorten the experiment, and so decrease this leakage, the eggs in later experiments on amino acid uptake were infected immediately after their preliminary starvation. With this method no allowance could be made for the inherent variation in uptake of different eggs; this decreased the sensitivity of the method.

Assessment of results. During these experiments chorio-allantoic membranes often became detached from the egg shells. This resulted in a big increase in glucose uptake and of leakage of ninhydrin-positive material into the medium. Because of this, all results on fluids from eggs with detached membranes have been excluded.

The significance of the effect of infection on glucose uptake was found by the 't' test, using the differences in uptake of glucose between days 2 and 4 for infected and uninfected eggs. For uptake of amino acids an analysis of variance was made, and the significance of the interaction factor for infection and amino acid addition calculated (Snedecor, 1946). As only one degree of freedom was associated with the interaction factor, the square root of the value of 'F' was taken and a table of 't' entered with this.

RESULTS

Effect of starvation on production of influenza B haemagglutinin

Eggs were de-embryonated, washed and starved for 24 hr. as described above. They were then infected with 0.5 ml. undiluted allantoic fluid and rotated for 1 hr. The eggs were then drained, rinsed-out and refilled with BSS alone, and some eggs given BSS + glucose 0.5 mg./ml. They were then rotated a further 24 hr. and the haemagglutinin titrated. In agreement with the results of

Daniels, Eaton & Perry (1952) it was found that only those eggs given glucose produced detectable haemagglutinin.

Infection of all the cells of the de-embryonated egg

To make any effects of infection as clear as possible it was desirable to infect all the exposed cells of the chorio-allantoic membrane. To test for this attempts were made to grow influenza A virus in eggs previously infected with influenza B virus. Cells previously infected with influenza B virus do not support the growth of influenza A virus (Henle, 1953); therefore, when eggs after infection with influenza B virus and super-infection with influenza A virus gave detectable influenza A haemagglutinin, this indicated that not all the cells had been infected with influenza B virus. In performing the experiment eggs were de-embryonated, starved 24 hr. then rotated 24 hr. with BSS+glucose (0.5 mg./ml.). They were then drained, refilled and infected with influenza B virus. At different times the eggs were drained, refilled and infected with influenza A virus (0.5 ml. undiluted allantoic fluid). After 1 hr. the eggs were drained and refilled and excess antiserum (prepared in rabbits) to influenza B virus added. The eggs were rotated 24 hr., and the haemagglutinins were checked for specificity against antiserum to influenza A virus. Controls were infected with influenza B virus only (+ normal serum) or with influenza A virus only. The results are shown in Table 1. The presence of normal serum had no

Table 1. *The time of exposure to influenza B necessary to prevent the growth of superinfecting influenza A in the de-embryonated eggs*

Antiserum to influenza B was added after the cells had been exposed to the influenza B inoculum for the time shown and then superinfected with influenza A for 1 hr. It was sufficient to neutralize all the influenza B liberated from the cells. Antiserum to influenza A neutralized 4 haemagglutination (HA) units influenza A at a dilution of 1/320; antiserum to influenza B neutralized 4 HA units influenza B at a dilution of 1/160. Neither antiserum neutralized the heterologous strain at a dilution of 1/40.

Egg no.	Additions to eggs			Period of adsorption for flu B virus (hr.)	Final haemagglutinin titre	Haemagglutinin after exposure to flu A antiserum
	Flu A virus	Flu B virus	Antiserum to flu B			
Experiment 1						
1	+	+	+	2	128	<4
2	+	+	+	2	128	<4
3	+	+	+	2	32	<4
4	+	+	+	4	128	<4
5	+	+	+	4	16	<4
6	+	+	+	4	32	4
7	-	+	-	4	32	.
8	-	+	-	4	32	.
9	-	+	-	4	128	.
10	+	-	+	.	128	.
11	+	-	+	.	128	.
Experiment 2						
1	+	+	+	24	<4	.
2	+	+	+	24	<4	.
3	-	+	-	24	16	.
4	-	+	-	24	8	.
5	+	-	+	.	16	.
6	+	-	+	.	16	.

effect on the amount of haemagglutinin produced. A 4 hr. infection period with 0.5 ml. undiluted influenza B virus containing $10^{9.7}$ infectious doses/ml. did not infect all the cells since influenza A virus subsequently grew. Exposure for 24 hr. (in which time several cycles of virus multiplication were possible) to 0.1 ml. undiluted influenza B virus was usually sufficient to prevent detectable production of influenza A haemagglutinin; hence a 24 hr. infection period was adopted for the work on uptake of glucose and amino acids. In these conditions an inoculum of haemagglutination titre 1024 and infectivity in the allantoic sac of 10^8 egg infective doses (EID)/0.1 ml. gave rise to fluid in the de-embryonated egg of haemagglutination titre 32 and infectivity 10^7 EID/0.1 ml.

Effect of infection on the uptake of glucose

Early experiments showed that there was no detectable production of reducing substances in the de-embryonated egg in absence of glucose. When uptake of glucose by normal and infected eggs was measured it was found that infection increased uptake (Table 2). The effect was quite small but was statistically significant ($0.02 < p < 0.05$ in the experiment shown) and was obtained in each of four experiments.

Table 2. *Increased uptake of glucose in de-embryonated eggs after infection with influenza B virus*

Initial glucose concentration was 2.4 mg./5 ml. Fluids in the eggs were changed daily. For the first day no glucose was present. Eggs were infected after 2 days.

Egg no.	Infection with flu B virus	Haemagglutination at		Glucose (mg./5 ml.) recovered at		Difference (2-4 days)
		3 days	4 days	2 days	4 days	
1	+	4	8	1.85	1.94	-0.09
2	+	8	16	1.80	1.76	+0.04
3	+	8	16	1.98	1.78	+0.20
4	-	.	.	1.66	1.85	-0.19
5	-	.	.	1.58	2.02	-0.44
6	-	.	.	1.64	1.84	-0.20

Effect of infection on the uptake of amino acids

Work on the uptake of amino acids was made more difficult by a continuous and variable leakage of ninhydrin-positive material from the cells into the experimental fluid. Deproteinizing followed by boiling for 30 min. in 2 N-NaOH decreased the amount of ninhydrin-positive material only by 20%. It seems likely that the remaining 80% was largely made up of amino acids (amino acids were shown to be present by paper chromatography of the de-salted fluid) but this has not been definitely established. Because of this leakage, eggs given BSS unsupplemented with amino acids were used as controls in all experiments. Amino acid uptake was defined as 'amino acid concentration initially present + average concentration of ninhydrin material finally present in control eggs - average concentration of ninhydrin-positive material finally present in experimental eggs'.

Uptake of amino acids given singly. No uptake of glycine, serine, phenylalanine, lysine, tryptophan or alanine (initial concentration 10 μg . α -amino-N/ml.) by infected or uninfected eggs was found.

Uptake of mixtures of amino acids. Casein hydrolysate (Allen & Hanbury 'vitamin free') at an initial concentration of 100 μg ./ml. was taken up readily. Uptake ranged from 35 to 100 % in different experiments; it was not affected by infection with influenza B virus. A defined mixture of amino acids was tried next. Eagle (1955*a, b*) found that 13 amino acids were required for the growth of several strains of animal cells in tissue culture, so these amino acids were used. For convenience of estimation their concentrations were half those required for optimal growth of cells of the human epidermoid carcinoma HeLa (Eagle, 1955*a*). Most of the amino acids were available only as the DL-forms so the quantities of these were doubled to maintain the concentrations of the L-forms at the desired concentrations. (Eagle, 1955*a*, found that the D-amino acids had no effect on cell growth.) Good uptake of this mixture was found but there was no difference in the uptakes of infected and uninfected cells.

Because of the good uptake of the defined mixture of amino acids, the effect of omitting single amino acids from it was tested. When cysteine, histidine, or methionine was omitted the remaining amino acids were taken up better by uninfected than by infected cells (Table 3). For each amino acid the effect was highly significant ($p < 0.001$ for the combined experiments). Omission of any other amino acid had no significant effect on amino acid uptake. For each amino acid omitted (except isoleucine) different experiments gave both bigger and smaller uptakes by infected eggs than by uninfected eggs. When isoleucine was omitted, in three separate experiments, uptake was bigger by uninfected than by infected eggs, but the difference was small.

Action of DL- β -phenylserine and DL-ethionine on growth of influenza B in de-embryonated eggs

Ackermann (1951*b*) reported ethionine, and Dickinson & Thompson (1957) β -phenylserine, to delay growth of influenza A in isolated chorio-allantoic membrane and to be antagonized by the analogous natural amino acids (methionine and phenylalanine, respectively). In view of the results obtained here on amino acid uptake it was of interest to test these two compounds for ability to delay growth of influenza B in de-embryonated eggs. DL-ethionine was obtained from Ashe Laboratories Ltd., Leatherhead, Surrey; DL- β -phenylserine was made in our laboratories.

After 24 hr. starvation, de-embryonated eggs were filled with 10 ml. BSS + 1 mg. glucose/ml., infected with 0.1 ml. undiluted influenza B and rotated for 1 hr. The eggs were then drained, rinsed-out and refilled with BSS + glucose + 2 mg./ml. of the amino acid analogue. The eggs were then rotated and haemagglutinin was titrated at 24 hr. intervals. In these conditions DL-ethionine delayed influenza growth markedly (Table 4) but DL- β -phenylserine did not. The activity of DL-ethionine was annulled by DL-methionine, 1 mg./ml.

Table 3. *The uptake of a mixture of 13 amino acids by infected and uninfected cells of the de-embryonated egg and of similar mixtures with one amino acid omitted*

The complete amino acid mixture contained ($\mu\text{g. } \alpha\text{-amino-N/ml.}$): L-arginine, 0.35; L-cysteine, 0.17; L-glutamine, 3.5; DL-histidine, 0.35; DD-isoleucine, 1.4; DL-leucine, 1.4; DL-lysine, 1.4; DL-methionine, 0.35; DL-phenylalanine, 0.7; DL-threonine, 1.4; DL-tryptophan, 0.14; L-tyrosine, 0.7; DL-valine, 1.4.

Ninhydrin-positive material ($\mu\text{g. } \alpha\text{-amino-N/ml.}$)							
Amino acid omitted	Initial	Average final		Average uptake†			P*
		Uninfected eggs	Infected eggs	Uninfected eggs (U)	Infected eggs (I)	Difference (U - I)	
None	0	9.0	14.5	8.6	11.6	-3.0	c. 0.45
	13.3	13.7	16.2				
None	0	5.2	6.0	5.3	2.0	+2.0	c. 0.3
	13.3	13.2	17.3				
Cysteine	0	3.5	7.1	6.5	3.0	+3.5	<0.02
	13.1	10.2	17.3				
Cysteine	0	4.5	7.2	7.9	3.9	+4.0	<0.02
	13.1	9.7	16.4				
Cysteine	0	8.9	9.6	9.4	5.4	+4.0	c. 0.4
	13.1	12.6	17.3				
Histidine	0	5.8	7.9	9.6	4.2	+5.4	<0.05
	13.0	9.2	16.7				
Histidine	0	6.1	5.6	9.0	-1.7	+10.7	<0.01
	13.0	10.1	20.3				
Histidine	0	6.0	8.2	9.0	4.5	+4.5	c. 0.2
	13.0	10.0	16.7				
Methionine	0	7.2	5.9	6.5	0.4	+6.1	<0.01
	13.0	13.7	18.5				
Methionine	0	5.2	5.9	8.6	3.9	+4.7	c. 0.25
	13.0	9.6	15.0				
Methionine	0	5.4	7.9	5.0	1.5	+3.5	<0.02
	13.0	13.4	19.4				

* P, significance of difference in uptake by normal and infected eggs.

† Uptake was defined as 'initial amino acid concentration + average final concentration of ninhydrin-positive material in control eggs - average final concentration of ninhydrin-positive material in experimental eggs'.

Table 4. *The action of DL-ethionine and DL- β -phenylserine on growth of influenza B virus in the de-embryonated egg*

Experiment 1				Experiment 2		
Egg no.	DL-Ethionine (2 mg./ml.)	HA*		Egg no.	DL- β -Phenylserine (2 mg./ml.)	HA* at 24 hr.
		20 hr.	44 hr.			
1	+	<4	8	1	+	64
2	+	<4	8	2	+	32
3	+	<4	.†	3	-	16
4	+	<4	.	4	-	8
5	-	16	.	5	-	8
6	-	32	.			
7	-	16	.			

* HA = haemagglutination titre.

† Not tested.

DISCUSSION

To make any effect of infection on cell metabolism as clear as possible, a uniform population of cells, all of which are infected, should be used. This ideal has only partly been realized: the cells of the de-embryonated egg directly exposed to the experimental fluid are known to be very uniform (Henle, 1953), and all exposed cells were infected, but the underlying cells may have contributed to amino acid and glucose uptake. However, the increased uptake of glucose and increased leakage of ninhydrin-positive material when both sides of the chorio-allantoic membrane are exposed (when it becomes detached from the shell) suggest that this contribution is normally small. Cairns & Fazekas de St Groth (1957) estimated that there were 3×10^7 allantoic cells lining the chorio-allantoic membrane of the 15-day egg. This number implies that in the present work exposure to 100 egg infectious doses of virus/cell for 4 hr. was insufficient to infect every cell. Use of several cycles of virus multiplication to infect every cell made it impossible to relate effects on uptake to specific stages of virus development, but did not make these effects any less real.

Infection of the cells of the de-embryonated egg decreased the uptake of mixtures of amino acids from which cysteine, histidine or methionine had been omitted. This suggested an increased need of the infected cell for these three amino acids. The observed decrease in uptake could not be due to exhaustion of cells producing virus in unphysiological conditions: no difference in uptake was obtained when the complete mixture was used or when any one of the other ten amino acids was omitted, and infection stimulated glucose uptake. The interpretation of the results as an increased need by infected cells for cysteine, histidine and methionine is strengthened by the inhibition of influenza growth in the de-embryonated egg by the methionine analogue ethionine, but not by the phenylalanine analogue β -phenylserine. The lack of activity of β -phenylserine in the de-embryonated egg, though consistent with the results on amino acid uptake, is surprising since the de-embryonated egg is of the same tissue as the isolated chorio-allantoic membrane (in which the analogue is a powerful inhibitor of virus multiplication.) The reason for this difference is not known; Mrs M. J. Thompson (personal communication) has shown that it is not due to the virus strain used, the age of the tissue, the preliminary starvation, or the culture medium. Although results obtained in a tissue culture system need not apply to the intact animal, the results reported here suggest that it might be worth while to examine analogues of cysteine, histidine and methionine for ability to inhibit influenza growth.

I wish to thank Dr Lois Dickinson for advice and criticism throughout this work, Dr M. R. Gurd for advice on the statistical analysis, Mr P. Oxley for a supply of DL- β -phenylserine, Dr G. Woolfe and Mr C. E. Coulthard for their interest, and Miss M. L. Rose and Miss J. M. Simpson for technical assistance.

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Studies on the Bacterial Component of Soybean Root Nodules: Cytology and Organization in the Host Tissue

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SUMMARY: Light- and electron-microscope studies of bacteroids in soybean nodule sections and suspensions of isolated bacteroids have shown well-defined nuclear elements, containing tangled filaments and dense granules. The cytoplasm was poorly stained as seen in the light microscope but had considerable dye-reducing activity localized at the poles, giving the appearance of granules. However, electron micrographs revealed no electron dense granules in the cytoplasm near the poles. Cell walls were not found on bacteroids in suspensions isolated from nodules, but were present on bacteria grown *in vitro*, and on bacteroids in the host tissue. Electron micrographs of thin sections of mature nitrogen-fixing nodules revealed a system of double membranes enclosing groups of four or six bacteroids. These membranes appeared to originate from the host cells and their development was followed in nodules aged from 1-5 weeks; they could be centrifugally separated from the bacteroid fraction of crushed nodules. The origin of the membrane system and its possible role in nodule functions is discussed.

In continuing the study of the bacterial component of soybean root nodules, the term 'bacteroid' is again used to denote the non-proliferating bacteria found in large numbers in the cytoplasm of the cells of the central tissue of mature nitrogen-fixing nodules; earlier proliferating stages in developing nodules are referred to simply as bacteria (Bergersen, 1958). This paper reports light- and electron-microscope studies of the bacteroids and their organization in nodule cytoplasm. Nodules of various ages are also examined in order that the development of the bacteroids and their attendant structures might be followed. The main features of the findings are illustrated by selected electron micrographs and diagrams.

METHODS

Plant and bacterial material. Plants were grown in the same conditions as before and nodules, cultures, and bacteroid suspensions were prepared as described by Bergersen (1958).

Light microscopy. Bright field microscopy was done with Wild 'Fluotar' objectives and for phase-contrast microscopy of unstained sections and bacteroid suspensions, the Wild 'Varicolor' system was employed (Bergersen, 1957). Microtome sections of nodules were examined unstained or stained by the rose bengal + light green method (Brenchley & Thornton, 1925). Bacterial preparations were examined mounted in M/15 phosphate buffer (pH 7.0), unstained, by phase contrast; they were stained with Janus green B (0.005%, w/v) and tetrazolium blue (BT, 0.1 mg./ml.) for the demonstration of reducing

sites. Nuclear material in the bacteria and bacteroids was stained by the HCl-Giemsa method (Robinow, 1942) after fixation in 1% (w/v) osmic acid in phosphate buffer (pH 7.0) followed by 3 min. hydrolysis in N-HCl at 60°. Basic fuchsin 1% (w/v) in 5% (w/v) phenol in water was also found useful as a nuclear stain for bacteroids, no hydrolysis being required. Lipid material was stained by Sudan black B (0.25%, w/v) in ethylene glycol. Cell walls were stained with 1% (w/v) crystal violet after 20 min. treatment with 10% (w/v) tannic acid.

Electron microscopy. Material for electron microscopy was prepared as follows.

(a) *Nodules.* These were cut into strips 0.5–1.0 mm. wide and 0.5–1.0 mm. thick, and quickly immersed in cold 1% (w/v) osmic acid in M/15 phosphate buffer (pH 7.0). After 4 hr. fixation, the tissue pieces were washed in distilled water and dehydrated through a graded ethanol+water series into absolute ethanol, followed by two changes of *n*-butyl methacrylate monomer, and two changes of partially polymerized methacrylate, then finally polymerized at 70° for 2 hr. Thin sections were cut with a Farrant & Powell (1956) thermal expansion microtome, mounted on collodion-coated copper grids, and examined in an R.C.A. model EMU-8B electron microscope, micrographs being made at magnifications between 5000 and 12,000 diameters followed by further photographic magnification.

(b) *Bacteria.* Suspensions of bacteroids from crushed nodules, prepared as previously described (Bergersen, 1958) and bacteria from culture were centrifuged and re-suspended in M/15 phosphate buffer (pH 7.0) containing 1% (w/v) osmic acid. The cells were then centrifuged down, washed in water to remove excess osmic acid and then dehydrated by centrifugation and re-suspension in 30, 60, 90% (v/v) ethanol in water and finally in absolute ethanol: the pellet was then embedded by the procedure described above.

RESULTS

Structure and cytology of bacteroids from nitrogen-fixing nodules

Light microscopy. The bacterial component (bacteroids) of nitrogen-fixing nodules consisted of rods ($3\text{--}5 \times 0.5 \mu$) with slightly swollen ends which gave them a club-shaped appearance. Bacteria of the same strain grown *in vitro* had similar dimensions, but were parallel sided. The features of the bacteroids seen by the various staining procedures are summarized in Fig. 1. The nuclear material was in two forms: (i) discrete, more or less spherical bodies surrounded by the unstained perinuclear area (Bergersen, 1955); (ii) elongated, less intensely staining bodies, occupying half to two-thirds of the length of the cells in an axial position. The perinuclear area was diminished or absent with the latter type of nucleus. In all cases the cytoplasm stained poorly with basic dyes and with Janus green B and BT except for localized areas near the poles of the cells, where Janus green B was rapidly reduced through the pink to the leuko form and blue formazan was rapidly deposited from BT. In the case of BT this effect became more pronounced when sodium succinate or sodium

glutamate was added to the buffer at a concentration of 0.02 M. These active polar areas had the appearance of granules and were stained by Sudan black B and osmic acid. They were similar in size to granules with the same activities seen in clover nodule bacteroids (Bergersen, 1955).

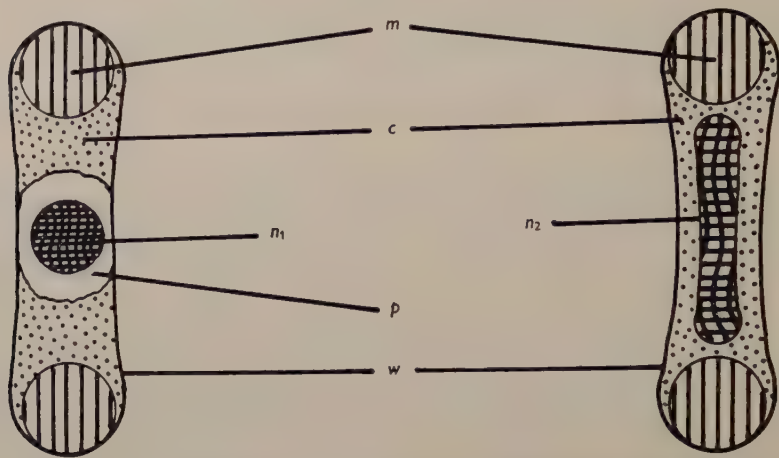


Fig. 1. The cytological features of the two types of bacteroids separated from mature, nitrogen-fixing soybean root-nodules as seen by light microscope methods. *c*, cytoplasm with low affinity for stains; *w*, cell margins unstained by cell-wall stains, but having osmiophilic properties; *m*, polar regions of reducing activity with respect to janus green B, and tetrazolium dyes; *n*₁, intensely staining chromatinic material surrounded by an unstained perinuclear area, *p*; *n*₂, elongated less intensely stained nuclear material.

Electron microscopy. Bacteroids seen in thin sections of nodule tissue or thin sections of embedded suspensions isolated from crushed nodules, possessed clearly defined nuclei whose shape and position agreed closely with the chromatinic bodies stained in whole cells by the HCl-Giemsa method and seen in the light microscope. In detail the nuclei consisted of intertwined filamentous material and several dense granules in an electron transparent matrix, but without any limiting membrane (Pl. 4, fig. 5). In the elongated nuclei the filaments were fine and loosely interwoven, while in the compact nuclei, which are not illustrated, they were thicker and very tangled. Along the latter type of nucleus appeared a diffuse osmiophilic or electron dense region corresponding to the position of the non-staining perinuclear areas. The cytoplasm of the bacteroids was vacuolated and there were no structurally recognizable polar granules: instead there appeared to be spaces in the cytoplasm at the positions occupied by the dye-reducing areas described in the previous section (Pl. 4, fig. 5; Fig. 1). This may have been due to the presence of electron-transparent material or loss of material during embedding as suggested by Chapman (private communication) in relation to the 'peripheral bodies' of *Bacillus cereus* described by Chapman & Hillier (1953). Bacteroids in suspensions isolated from crushed nodules had no cell wall, the cytoplasmic boundary simply had a sharply defined margin. However, in nodule sections a mem-

branous wall surrounded each bacteroid: sectioned bacteria from culture also possessed cell walls (Pl. 4, figs. 6, 7).

In the sections of the younger nodules the bacteria had denser unvacuolated cytoplasm except in the polar positions where small vacuoles appeared, and the nucleus was usually elongated, the filaments again being plainly visible (Pl. 2, fig. 3). In nodules aged from 1 to 2 weeks the bacteria were still actively dividing (Bergersen, 1958), and at 3 weeks bacteroid formation was about complete. No marked cytological changes were seen at this point in the present study: this is in contrast to the formation of bacteroids in subterranean clover nodules (Bergersen, 1955) where changes in shape and nuclear arrangement were readily seen.

The organization of bacteroids in mature, nitrogen-fixing nodules

In microtome sections of nodules examined in the light microscope, the bacteroids were apparently randomly packed into the cytoplasm in the host cells. Remnants of host nuclei were still visible and there were several small vacuoles in the host cytoplasm of bacteroid-containing cells. Electron micrographs of thin sections of similar nodules revealed the bacteroids lying within a membrane system in the host cytoplasm. Individual bacteroids lying within their cell walls in groups of two, four or six were surrounded by a membrane envelope (Pl. 1), whose double-layered structure is seen in Pl. 4, fig. 8. In most preparations there was a certain amount of shrinkage of the bacteroids resulting in empty spaces between them and their cell walls; it is probable that in life these were closely applied to the surface of the bacteroids.

The space between the bacteroid walls and the membrane envelope was occupied by diffuse or slightly granulated material. The host cytoplasm between the enclosed groups of bacteroids was composed of rather diffuse granular material with numbers of structures interpreted as tubules cut in various planes (Pl. 1). The significance of these will be discussed below.

The organization of the bacteria in developing nodules

In order to follow the development of the membrane system, tissue from nodules aged from 1 to 5 weeks was examined by light and electron microscopy. Bacterial numbers were low in infected cells of one week old nodules and slender infection threads could be seen with the light microscope in the majority of host cells of the central tissue. These threads traversed dividing cells whose nuclei were in various stages of mitosis, and free bacteria were also seen in dividing host cells. Infection threads were difficult to find in the cells of 2 weeks or older nodules, although many dividing cells containing bacteria were seen. It seemed, therefore, that initially the bacteria were distributed through the dividing cells of the young developing nodule by ramifications of infection threads from which the bacteria were released into the cytoplasm of the cells, the bacteria being continuously re-distributed by division of the host cells containing them. Host cell division became less frequent and finally ceased after about 2 weeks from the first appearance of the nodule, while the

bacteria then multiplied until the swelling host cells were fully packed with them.

Electron micrographs of thin sections of 1-week-old nodules were obtained which contained portions of infection threads (Pl. 2, fig. 2). These were enclosed in well-defined double membranes, inside which was a layer of material presumed to be the cellulose walls of the thread (McCoy, 1932; Schaede, 1940, 1941). Within these walls the cell wall of the bacteria could be seen, separated by the embedding procedures from the cell surface. A number of sections of 1-week-old nodules showed single, isolated bacteria, few in number and enclosed in the double membranes. In other sections the bacteria, similarly enveloped, appeared in a group near the host-cell nucleus (Pl. 2, fig. 3; Pl. 3, fig. 4).

In electron micrographs of sections of 2-week-old nodules the bacteria were present in greatly increased numbers, each enclosed by a membrane. In nodules aged 3 weeks individual bacteria were enclosed in groups of two or sometimes four bacteria within the envelope; 4- and 5-week-old nodules contained the structures of the mature nodules described in the previous section. In all micrographs of 2- to 5-week-old nodules the sectioned cytoplasmic tubules mentioned previously could be readily found. They were particularly plentiful in nodules aged 3 weeks, scattered in the cytoplasm between the membranes.

During the development of the bacteroids and their attendant membranes the host cytoplasm also changed considerably. The cytoplasm of the young host cells was typical of plant meristem (Heitz, 1957; Lance, 1957) and contained normal mitochondria, scattered Golgi regions and many ribonucleoprotein granules (RNP) some of which were associated with the membranes of the endoplasmic reticulum. With increasing age, the cytoplasm became less dense and RNP and Golgi regions disappeared, mitochondria became scarce and were confined to the cell periphery, and endoplasmic reticulum was much reduced. The appearance of the nucleus was normal up to three weeks. These changes were attendant on large increases in host cell volume, as seen in light microscopy of microtome sections of nodules.

Isolation of the membranes

The prominence and relative abundance of the membrane system described in previous sections, and the possibility that it may be concerned in processes vital to the functioning of the nodules, prompted an attempt to isolate samples of it from crushed nodules. Light microscope examination of supernatant fluids after centrifugation of bacteroids, showed numbers of sack-like bodies visible with the 'Varicolor' phase equipment when using green light of about 520 m μ wavelength. It was then found that by prolonged centrifugation at 6000 g of the filtered crushed nodules, these sack-like bodies were sedimented as a grey brown layer immediately overlying the bacteroids in the centrifuge tube. Such a preparation was made, the upper layer resuspended in M/15 phosphate buffer (pH 7.0) and re-centrifuged. After a further wash in buffer, the preparation was fixed in cold 1 % (w/v) osmic acid in phosphate buffer, dehydrated,

embedded in methacrylate and sectioned as described. The electron micrograph of Pl. 4, fig. 8, shows sections of the membranes whose thickness of 125 Å corresponds to the thickness of membranes in sections of intact nodules, and whose two-layered structure is clearly demonstrated.

In view of the fact that bacteroids in suspensions from crushed nodules were largely devoid of cell walls, the preparations described in this section probably contained bacteroid walls as well as membrane envelope materials. The thicker fragments are thought to be wall material (Pl. 4, fig. 8).

The cell walls of bacteria isolated from nodules of various ages

The observation that mature bacteroids were devoid of cell walls when isolated from nodules as suspensions may have a bearing on the failure of growth of bacteroids. Suspensions of bacteria from nodules aged from 1 to 6 weeks were prepared and stained by the tannic acid + crystal violet method. Bacteria from nodules aged up to 2 weeks possessed intact cell walls but from 3 weeks no cell walls could be stained.

DISCUSSION

The cytology of the bacteroids and of the proliferating bacteria from *in vitro* cultures presented no unexpected features. The chief structural difference between bacteroids and growing bacteria lay in the density of the cytoplasm of the latter compared with the vacuolated poorly staining cytoplasm of the bacteroids. In both types of cells the nuclear material consisted of intertwined filaments and dense granules, and no limiting membrane. The discrete spherical nuclei, however, seemed to be characteristic of the bacteroids and may represent nuclei incapable of further division, thus, perhaps, contributing to the non-proliferating nature of the bacteroids. The filaments of the nuclei are similar to filaments described in nuclei of *Salmonella* (Maaløe & Birch-Andersen, 1956), *Staphylococcus* (Bradfield, 1956) and *Escherichia coli* (Tomlin & May, 1955), to quote but a few examples.

The fact that bacteroids in suspension are largely devoid of their cell walls may also contribute to the failure of growth of bacteroids when placed in normal culture media (Almon, 1933). It is also interesting to note that the loss of bacterial dry weight reported previously when nodules are between 1 and 2 weeks old (Bergersen, 1958) may have been in part at least, due to loss of cell-wall material during preparation of the suspension from crushed nodules.

The method used for fixing and embedding nodule material for electron microscopy was arrived at after trials of a number of fixation times, osmic acid concentrations, temperatures, buffers and salt concentrations. The use of M/15 phosphate buffer (pH 7.0) as both buffer and salt medium with fixation in 1% osmic acid in the cold, gave best preservation of host cytoplasm and minimum shrinkage of bacteroids. The variation in osmotic pressure of the fixative had little effect on the extent of bacterial shrinkage, which probably occurred during dehydration.

A discussion of the organization of the bacteroids within the membrane system which has been described would be incomplete if no hypothesis of the

origin of the membranes were advanced. Figure 2 illustrates the author's proposals. It is suggested that the final organization results from outgrowths of the original infection thread, the outer membranes of which are pushed out before the bacteria as they grow out from the thread. This happens in host cells still undergoing division and connexion with the infection thread is soon

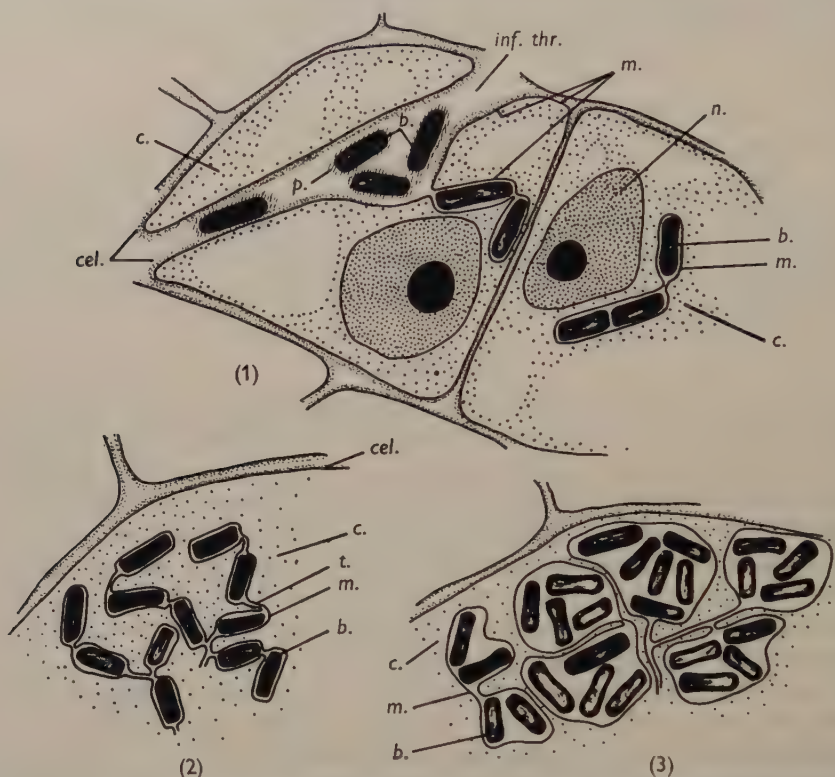


Fig. 2. Diagrammatic representation of the development of the membrane system enclosing the bacteroids in soybean root-nodules. (1) Bacteria escape from the cellulose-thickened infection thread, but are still enclosed by the thread membrane which is continuous with the host cell membrane. The host cell then divides and a small group of bacteria surrounded by the membrane is separated from the original infection thread by the developing cell wall. (2) Bacteria multiply within the enclosing membranes, the development of which keeps pace with bacterial multiplication, with the result that the bacteria become individually surrounded by inter-connected membrane envelopes. (3) The final stages in which the divisions of the bacteria result in groups within the enveloping membranes, firstly in pairs and ultimately in groups of four or six. *c.*, host cytoplasm; *cel.*, cellulose of host cell walls and lining infection threads; *inf.thr.*; *m.*, membrane envelope around bacteria, and initially continuous with infection thread membrane and host cell-membrane; *n.*, host nucleus; *b.*, bacteria; *t.*, interconnecting membrane tubule.

broken. The result is a distribution of bacteria in low numbers throughout the central tissue of the nodules, each bacterium surrounded by extensions of the thread membranes, seen as a membrane enclosing each cell. The bacterial growth which takes place, subsequent to cessation of host cell division, occurs within the membranes, growth of which keeps pace with bacterial multipli-

cation to give, at 2 weeks from the first visible appearance of the nodules, a large number of bacteria, each surrounded by inter-connected membranes. The sectioned tubules mentioned earlier represent the interconnecting portions of the system. The last few bacterial divisions occur in conditions where space is limited and the bacteria can no longer extend the tubules, with the result that the outer membrane is extended to form a sack, containing at first a pair of bacteria and later four or six bacteroids.

Nutman (1956) suggested that infection thread development is really an inward growth of the cell wall at the point of penetration. If this be so, the outer membrane of infection threads and the membrane envelope enclosing the bacteria may be regarded as being homologous with the host cell membrane. The dimensions of both (125 Å in thickness) are similar and this tends to support the hypothesis. One electron micrograph, which is not presented, has clearly shown that the thread membrane was continuous with the host cell membrane.

It is worthy of note that nitrogen fixation, in the system being studied in this work, begins when the nodules are between 2 and 3 weeks old and attains its maximum rate a week later (Bergerson, 1958). These times correspond to the final stages of development of the membrane system. It thus seems possible that this may be concerned in the organization of enzymes involved in the fixation of molecular nitrogen or in the assimilation of the reduced product into the metabolism of the host plant.

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EXPLANATION OF PLATES

Electron micrographs of thin sections of soybean nodule tissues, preparations of bacteroids and cultured bacteria, fixed in 1% (w/v) OsO₄ in M/15 phosphate buffer.

PLATE 1

Fig. 1. Mature bacteroid-containing cell of a 3-week-old nodule showing bacteroids (*b.*) in transverse section with their cell walls (*c.w.*) separated during preparation, from the cell surfaces. The bacteroids in groups of two, four and six are surrounded by a membrane envelope (*m.*) and the host cytoplasm (*c.*) is very diffuse and featureless. A number of sectioned tubules (*t.*) are associated with the membranes. Magnification, $\times 77,000$.

PLATE 2

Fig. 2. A section of a portion of an infection thread in a 1-week-old nodule; it runs through a strand of cytoplasm with vacuole (*v.*) on either side. The membrane (*m.*) around the thread is clearly visible and the grey lining of the thread is interpreted as the cellulose layer (*cel.*): this is absent from the portion on the right which is interpreted as the first stage in the movement of the bacteria (*b.*) from the infection thread into the host cytoplasm (see Fig. 2). Magnification, $\times 91,000$.

Fig. 3. A section of a portion of a cell of a 1-week-old nodule, in which individual bacteria enclosed in membranes (*m.*) are distributed throughout the cytoplasm of the host cell. The bacteria have dense cytoplasm and their nuclei contain filaments (*n.f.*). Magnification, $\times 136,000$.

PLATE 3

Fig. 4. A slightly younger cell than in Fig. 3: a group of individually enclosed bacteria are seen near the host nucleus (*h.n.*). The host cytoplasm is typical of young plant cells and contains numerous RNP granules (*r.n.p.*): endoplasmic reticulum (*e.r.*) and Golgi regions (*g.r.*) are present. Magnification, $\times 141,000$.

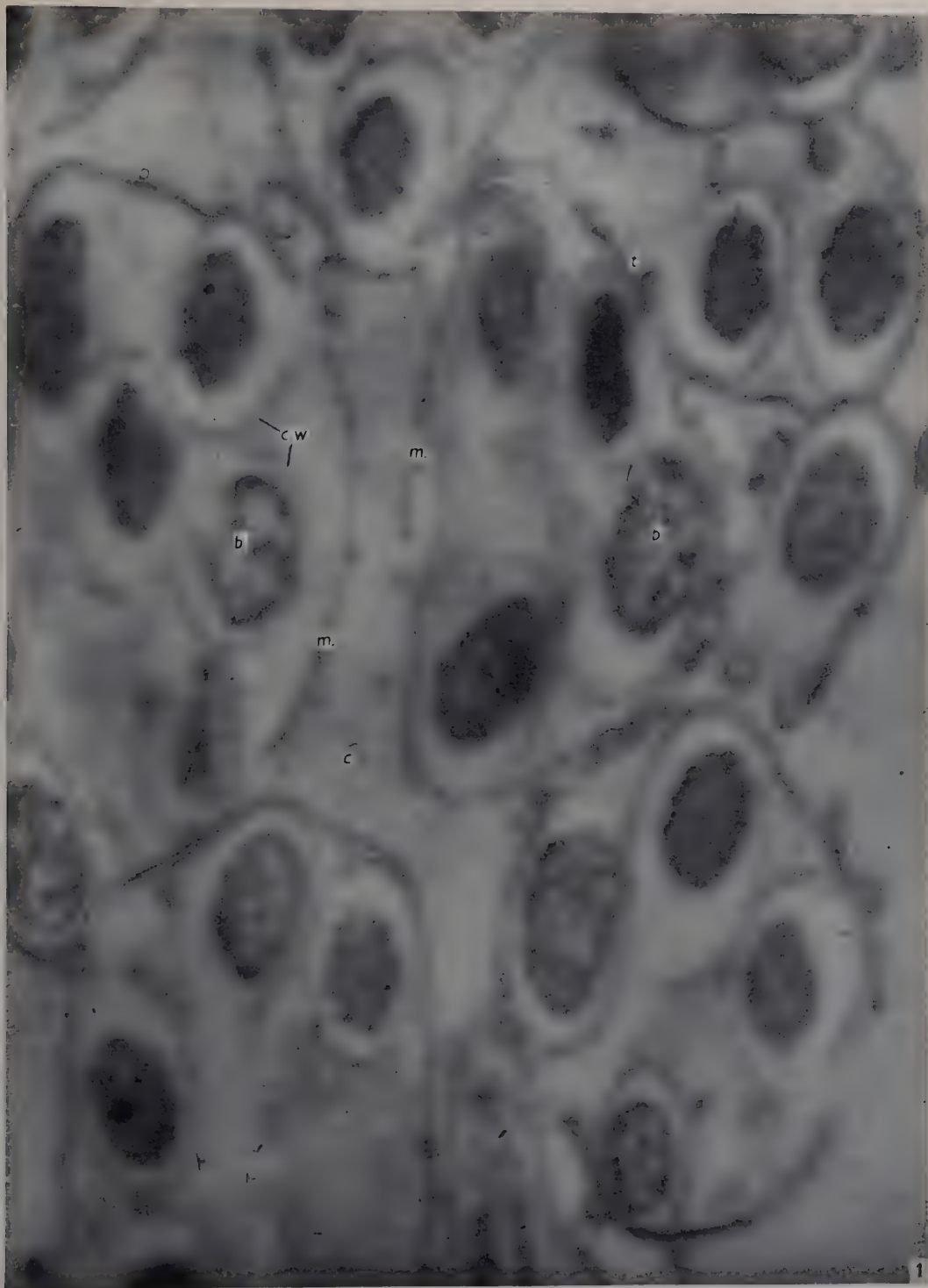
PLATE 4

Fig. 5. A longitudinal section of a bacteroid from a suspension isolated from 4-week-old nodules. Polar reducing sites (*r.s.*) appear as spaces in the cytoplasm and the axial nuclear material consists of fine filaments (*n.f.*) in an electron transparent matrix with two dense granules (*n.g.*). There is no cell wall. Magnification, $\times 116,000$.

Figs. 6, 7. *Rhizobium japonicum*, strain C.C. 711 grown on yeast + mannitol agar. Cell walls (*c.w.*) are present although separated from the cell surface, by shrinkage of the protoplast. The nuclei contain filaments (*n.f.*) and granules (*n.g.*). Magnification, $\times 77,000$.

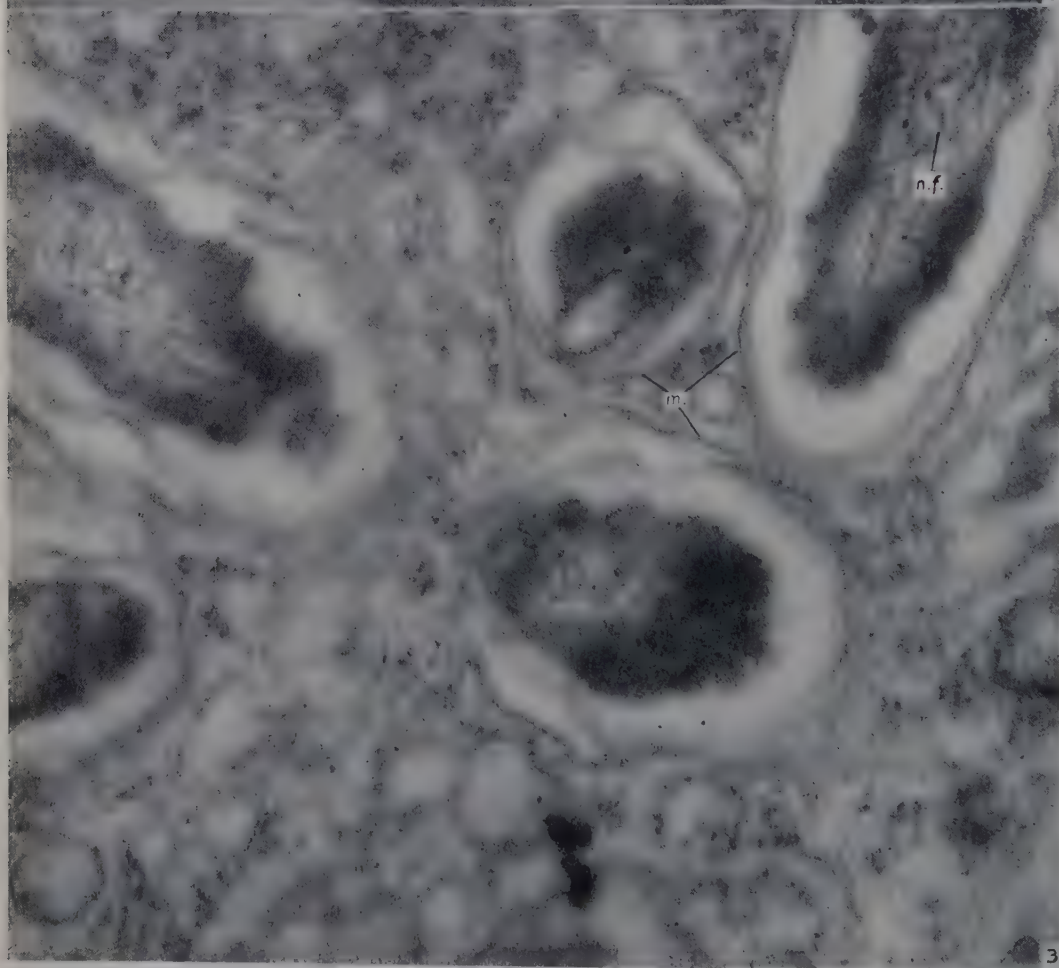
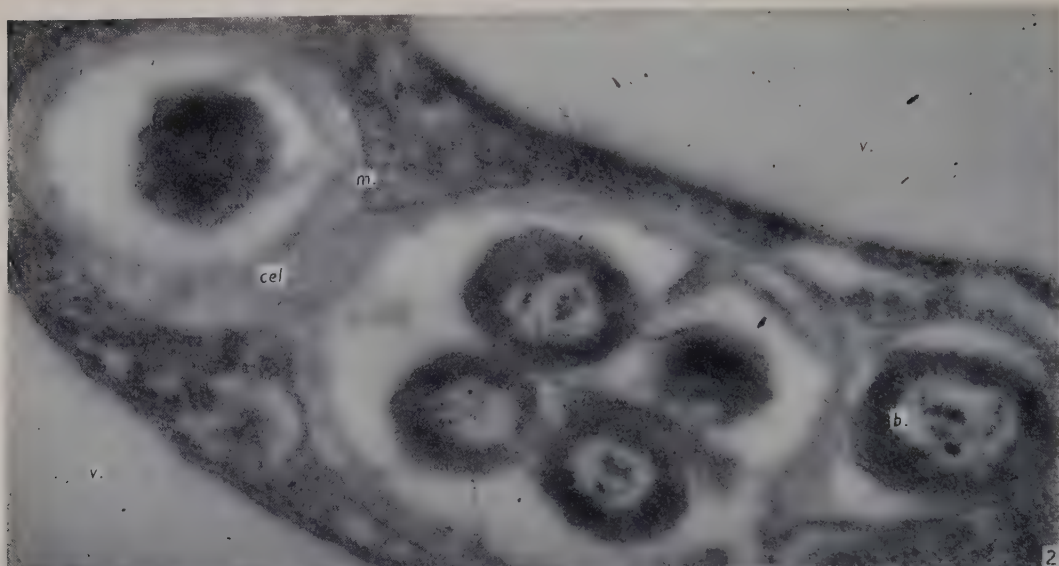
Fig. 8. Membranes isolated from crushed nodules. The double-layered structure is clearly shown (*m.*) and thicker fragments represent bacterial cell-wall material (*c.w.*). Magnification, $\times 119,000$.

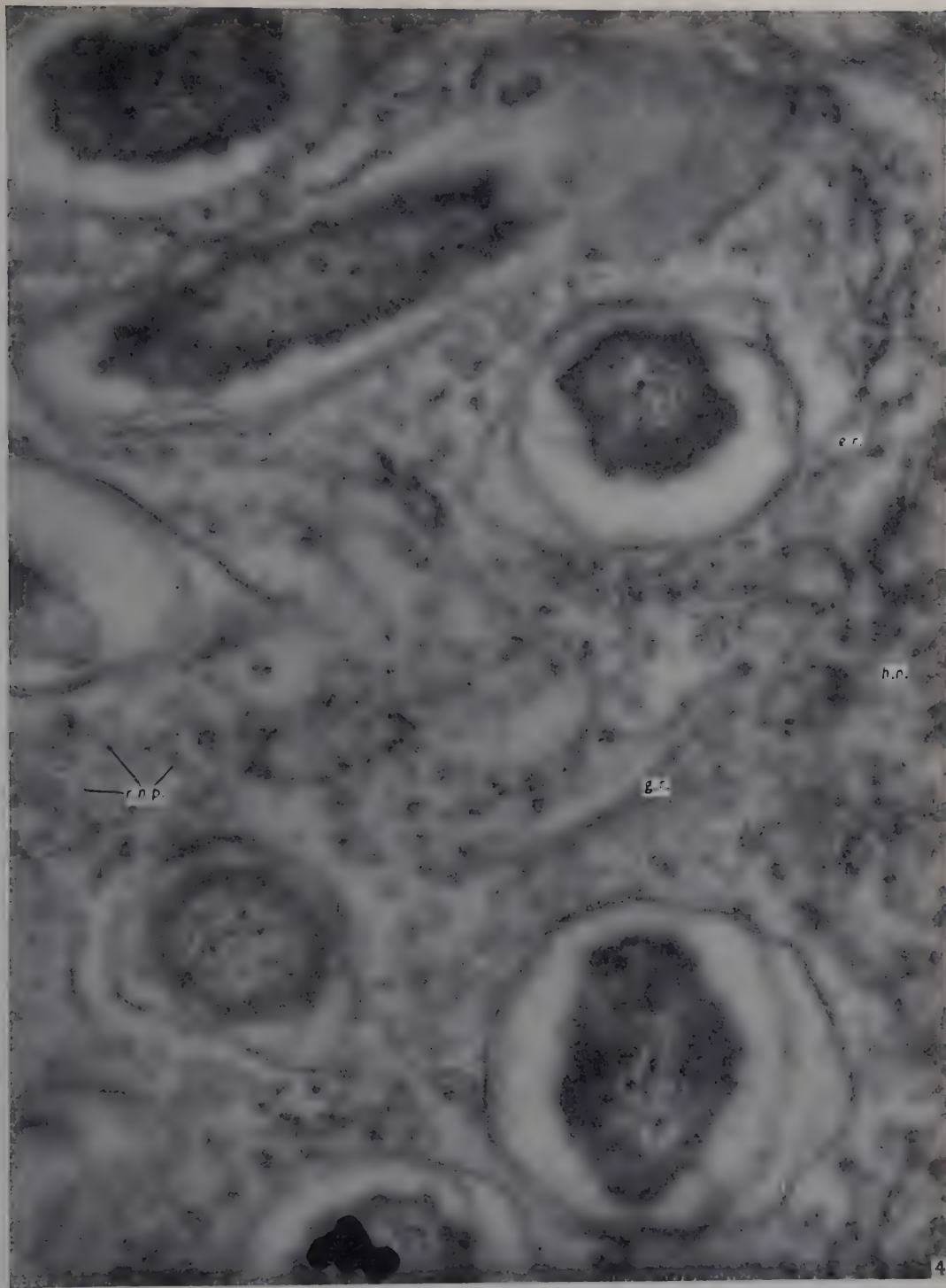
(Received 27 May 1958)



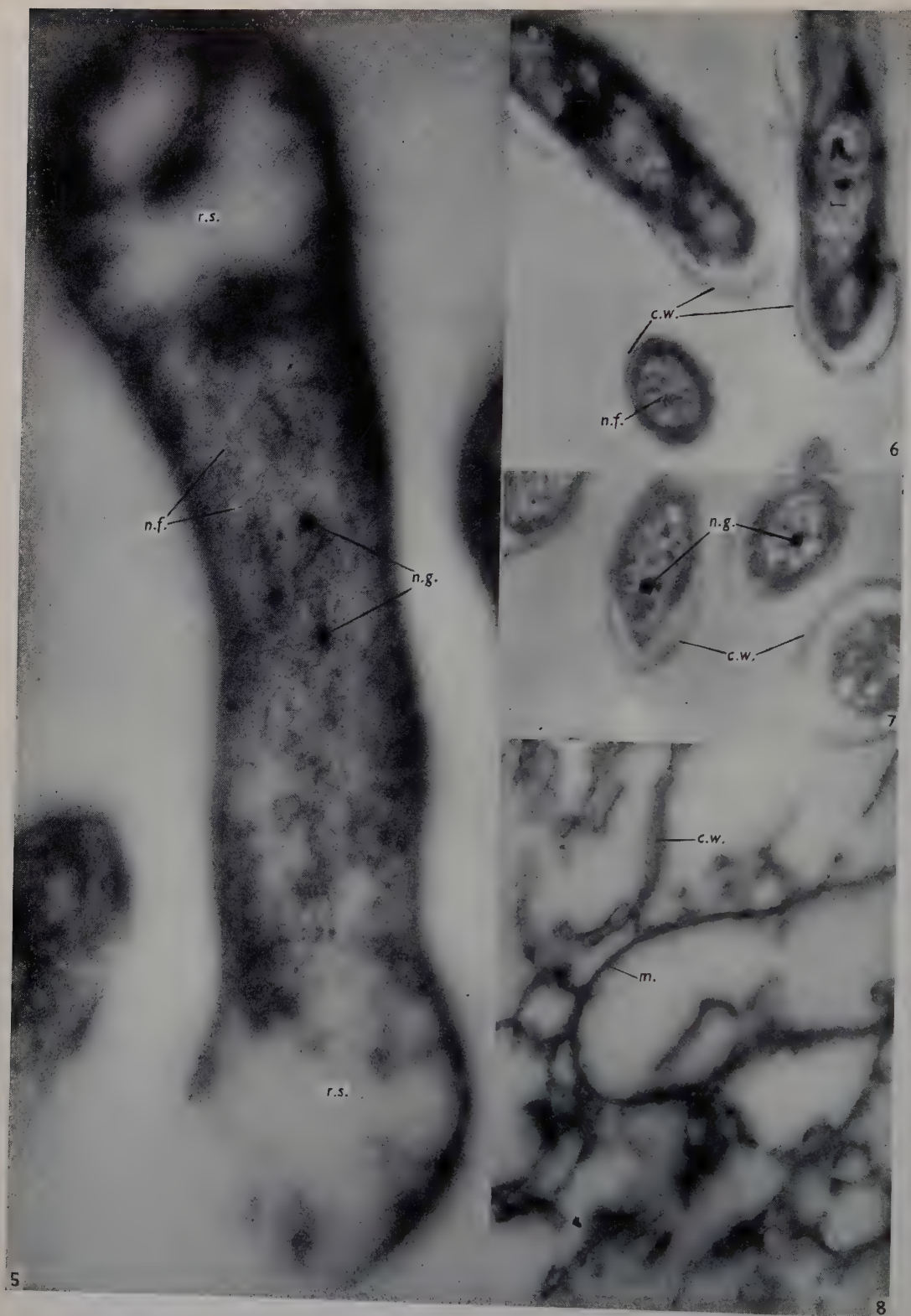
F. J. BERGERSEN & M. J. BRIGGS—THE BACTERIAL COMPONENT OF SOYBEAN ROOT NODULES. PLATE 1

(Facing p. 490)





F. J. BERGERSEN & M. J. BRIGGS—THE BACTERIAL COMPONENT OF SOYBEAN ROOT NODULES. PLATE 3



F. J. BERGERSEN & M. J. BRIGGS—THE BACTERIAL COMPONENT OF SOYBEAN ROOT NODULES. PLATE 4

The Globular Involution Forms of the Bifid Bacteria

By VERONICA SUNDMAN AND K. AF BJÖRKSTEN

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SUMMARY: The globular swellings known to appear occasionally in cultures of the bifid bacteria have been shown to occur in all known types of *Lactobacillus bifidus*. The swellings developed on a substrate which permitted abundant growth. The development of the globular involution forms was prevented by the addition of tryptic digest of cow's milk to the substrate. The similarity between the globular forms of *L. bifidus* and protoplasts is mentioned. The globular forms are considered to be organisms with incomplete cell walls which develop on a substrate adequate for growth but not for normal cell wall synthesis.

There are in the literature on the bifid bacteria (*Lactobacillus bifidus*) repeated notes on the occurrence of globular involution forms. Tissier (1900) stated that the whole organism can swell and give a bladder-like appearance as a response to acidity, extreme temperatures or inadequate nitrogen sources in the substrate. Orla-Jensen (1943) described his *Bacterium bifidum* strain no. 9 as extremely irregular with strongly clavate swellings. He assumed a relationship between morphology and physiological properties, and proposed as a possibility that the bladder-forming culture might represent a separate species. Negroni & Fischer (1944) published a micrograph of these involution forms of *L. bifidus* without more detailed descriptions of the conditions in which they occurred. In Negroni & Fischer (republished by Frank & Skinner, 1954) a developing involution globe is seen at no. 10 in the camera lucida drawing. Hayward, Hale & Bisset (1955), in a morphological study on *L. bifidus*, noted swollen and irregular organisms in some strains, and found a correlation between the abnormal morphology and sensitivity to oxygen. Olsen (1949) made an experiment to show whether the 'clubs' were a kind of resistant form, but could not find any increased heat resistance in cultures with many club-shaped organisms as compared with normal cultures.

Because of the pleomorphic character of the bifid bacteria, it may be difficult to decide whether a morphological feature is an involution form developed as a response to unfavourable surroundings, or belongs to the normal picture of the culture. The globular swellings dealt with in this paper are, with some experience, easily distinguished from the normally-occurring organisms with swollen ends. In its extreme form the involution bladder assumes a circular shape of 4 μ diameter, with a structure like a cross on its surface. The bladders are not easy to detect in ordinary stained smears, but are clearly seen in water mounts in the phase contrast microscope (Pl. 1, fig. 1). Compared with the specific amphora-like organisms seen in Pl. 1, fig. 2, and the swollen, bone-like organisms in Pl. 1, fig. 3, the globular involution bladders are more circular in shape and mostly larger than the normal cells.

ORGANISMS AND MEDIA

The strains studied were the same as in some other investigations (Gyllenberg & Carlberg, 1958; Sundman, af Björkstén & Gyllenberg, to be published) and included thirteen isolates of infant origin and *Lactobacillus bifidus* var. *pennsylvanicus* ATCC 11863. The strains are listed in Table 1, and grouped on the basis of nutritional demands according to Gyllenberg & Carlberg (1958) and in conformity with the classification of Dehnert (1957) which is based on fermentative, morphological and serological features.

Table 1. *Grouping of the strains of Lactobacillus bifidus investigated*

Strains studied	Nutritional type (Gyllenberg)	Group (Dehnert)
K ₁₇ , M ₁ , D ₁	A	IV
A ₁ , A ₃ , A ₄ , A ₆ , E ₁₅	B	V
B ₁₈ , C ₈ , C ₂₅	B	III
B ₄ , B ₁₁ , 11863	C	I-II

The stock cultures were kept in tomato agar stabs, incubated in a CO₂ atmosphere at 37° for 1-3 days and stored in the refrigerator for 7-14 days between the transfers. The tomato agar was a modification of the medium used by Petuely & Lynau (1954).

Table 2. *Composition of media*

	Tomato agar	G agar
Sodium acetate	1 g.	—
Ascorbic acid	—	1 g.
Dibasic potassium phosphate	0.5 g.	0.25 g.
Ammonium sulphate	0.4 g.	—
Casamino acids Difco	—	0.5 g.
Lactose	3.5 g.	3.5 g.
Cystein hydrochloride	0.04 g.	0.04 g.
Ca pantothenate	50 µg.	50 µg.
Biotin	0.5 µg.	0.5 µg.
Salts B*	0.5 ml.	0.5 ml.
Tween 80	0.5 ml.	0.5 ml.
Tomato agar Difco	1 g.	—
Yeast extract Difco	—	0.1 g.
Tryptic digest of cow's milk†	—	5 ml.
Human milk (skimmed)	—	2 ml.
Agar	1.5 g.	1.5 g.
Distilled water	100 ml.	100 ml.
pH value	6.6	6.6

* MgSO₄·7H₂O, 10 g.; FeSO₄·7H₂O, 0.5 g.; MnSO₄·H₂O, 0.35 g.; NaCl, 0.5 g.; distilled water, 250 ml.

† 100 ml. sterilized skimmed milk and 50 mg. trypsin (Gurr) incubated at 37° for 24 hr.

For morphological examination the medium of György (1953) supplemented with tryptic digest of cow's milk, human milk and agar was chosen. This medium is referred to below as G agar. The composition of media is given in Table 2.

RESULTS

All the strains studied grew well on both media in a CO₂ atmosphere. It was, however, noticed repeatedly that stab cultures in tomato agar, after vigorous growth during incubation, occasionally diminished their viability or lost it completely in a week in the refrigerator. In such cases the cultures could be restored by frequent transfers on G agar.

When the morphology of the bifid strains was continuously studied on G agar plates, it became evident that during the first 15 hr. the cultures belonging to the nutritional type B and Dehnert's group V (see Table 1) contained numerous globular swellings. Such a young culture is seen in Fig. 1. After incubation for one day the organisms assumed a more normal appearance and the globular swellings disappeared completely (Pl. 2, fig. 9). It was therefore assumed that the globular form either represented some early stage in the life cycle of the bifid bacteria or developed in the tomato agar stabs used as inoculum in the experiments. A closer examination of the stab cultures with the phase contrast microscope justified the latter alternative. All the strains studied produced globular swellings in the tomato agar. The globules were most numerous in the strains belonging to Dehnert's groups IV and V (see Table 1). The strains listed in the Table as type B, group III, as regards the number and size of the globules, had a position between the former strains and the strains of pennsylvanicus type (type C according to Gyllenberg; group I according to Dehnert) which showed more occasional and smaller globules.

The globular bladders could not be correlated with any growth phase of the cultures. They occurred on the tomato agar at every age of the culture, on plates as well as in stabs. On G agar no globules developed. The globular swellings on young G agar plates could all be attributed to the inoculum.

The micrographs in Pl. 1, figs. 4-7, represent the globule-containing growth on tomato agar of the different groups of strains as listed in Table 1. Each micrograph is representative for all the strains in the group since no morphological differences of significance could be detected within the groups (Sundman *et al.* to be published). The corresponding pictures of normal growth on G agar are seen in Pl. 2, figs. 8-11.

When the two media are compared, it seems obvious that the tomato agar, although permitting abundant growth, is deficient in some respect. Neither of the three components of the tomato agar which were not included in the G agar (Na acetate, (NH₄)₂SO₄, Difco tomato agar) induced globular swellings when added to the G agar. When, conversely, the G agar components (Difco caseamino acids, yeast extract, tryptic digest of cow's milk and human milk) were added separately to the tomato agar in the same concentrations as they occur in G agar and one of the most pronounced globule-forming strains (A₄) was grown on these substrates, it turned out that 5% (v/v) tryptic digest of cow's milk completely suppressed the globule formation in the tomato agar. The other components added had no influence on the development of the globular swellings. Additional experiments, where the tomato agar was supplemented with smaller amounts (3%, v/v, and 1%, v/v) of the tryptic digest and with

various quantities of untreated milk, respectively, showed that the untreated milk had no effect, nor had the addition of 1 % (v/v) tryptic digest; 3 % (v/v) tryptic digest was enough to suppress the globule formation to some degree, but the organisms still showed a tendency to swell.

DISCUSSION

During these experiments the strains showed no variation in their ability to grow on artificial media. The rate of growth was increased by frequent transfers, but no tendency to more aerobic growth was noticed. The globule formation was the same immediately upon isolation and after the strains had been kept for several months in the laboratory. Thus the observation by Hayward *et al.* (1955) that the swollen forms disappeared after prolonged artificial culture was not confirmed. Probably these authors worked with mixed cultures, the adaptation to aerobic growth suggests the presence of corynebacteria (cf. Gyllenberg, 1958).

The bladder-forming culture of *Lactobacillus bifidus* described by Orla-Jensen (1943) was an arabinose-fermenting strain. Orla-Jensen suggested that the strain might represent a separate species, with the peculiar globular cell morphology and the ability to ferment pentoses as characteristics separating it from other bifid bacteria. Orla-Jensen's observation was confirmed in the present work in so far as the most pronounced bladder formers were all found to ferment pentoses, either xylose only (Dehnert's group IV) or arabinose and xylose (Dehnert's group V). As, however, the globular swellings are to be found in all the strains studied, they cannot be used as a differentiating characteristic in a future separation of *L. bifidus* into different species or subtypes. On the contrary, the ability to form involution bladders on the media used in the present study may serve as a more general characteristic of all *L. bifidus* types, especially as the examination of a number of related organisms (Sundman *et al.* to be published) did not reveal the peculiar globular swellings in others except *L. bifidus*.

All types of bifid bacteria, independently of their basic growth requirements, are stimulated by the tryptic digest (personal communication from Dr H. Gyllenberg). It may be assumed that some peptide component in the cow's milk digest is required for normal growth. The morphological description of the *Lactobacillus bifidus* types by Dehnert (1957) does not contain any report on involution forms. The proteose peptone of the medium used, otherwise resembling our tomato agar, is assumed to have provided the peptide component necessary to prevent the development of involution bladders.

The bladder-forming tomato agar is not known to contain any growth-suppressing substances. The globular forms are therefore considered as the response of *Lactobacillus bifidus* to a nutritionally deficient environment. Lark (1958) reported the development of similar globular forms, induced by penicillin in a strain of *Alcaligenes fecalis*. The effect of penicillin lies in the blockage of the formation of new cell wall material (Park & Strominger, 1957). Whereas the lysozyme-induced protoplasts of *Bacillus megaterium* are formed as a result

of the attack of lysozyme on the Gram-positive cell wall (Weibull, 1953), the protoplast-like globules in *A. fecalis* are probably the consequence of the penicillin-blocked cell wall synthesis. In the case of the bifid bacteria, the synthesis of a normal cell wall seems to be more exacting than the other growth of the organism, and the protoplast-like bladders may appear as a result of a deficiency of the substrate. The known tendency of *L. bifidus* to show morphological variations when grown *in vitro*, being most regularly rod-shaped in the natural habitat, may point towards an exacting cell wall synthesis. The pleomorphic character of the bifid bacteria makes an exact description of their morphology rather difficult. Our observations indicate that special attention must be paid to the composition of the medium on which morphological observations for species characterization of the bifid bacteria are made.

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EXPLANATION OF PLATES

All photographs are of water-mounted smears of *Lactobacillus bifidus* strains grown at 37°. The phase contrast equipment of the Leitz Dialux microscope was used. Magnification, $\times 2500$.

PLATE 1

Fig. 1. Involution bladders. Strain A₆. Young surface culture (6 hr.) on G agar.
Fig. 2. Amphora-like organisms. Strain B₄. Surface culture (2 days) on G agar.
Fig. 3. Swollen, bone-like organisms. Strain M₁. Stab culture (3 days) in tomato agar.
Figs. 4-7. Tomato agar stab cultures (2 days) of the four different groups of strains as listed in Table 1 in the text. Fig. 4: strain K₁₇; fig. 5: strain A₄; fig. 6: strain B₁₈, fig. 7: strain B₁₁.

PLATE 2

Surface cultures on G agar at 37° of the four different groups of strains as listed in Table 1 in the text.

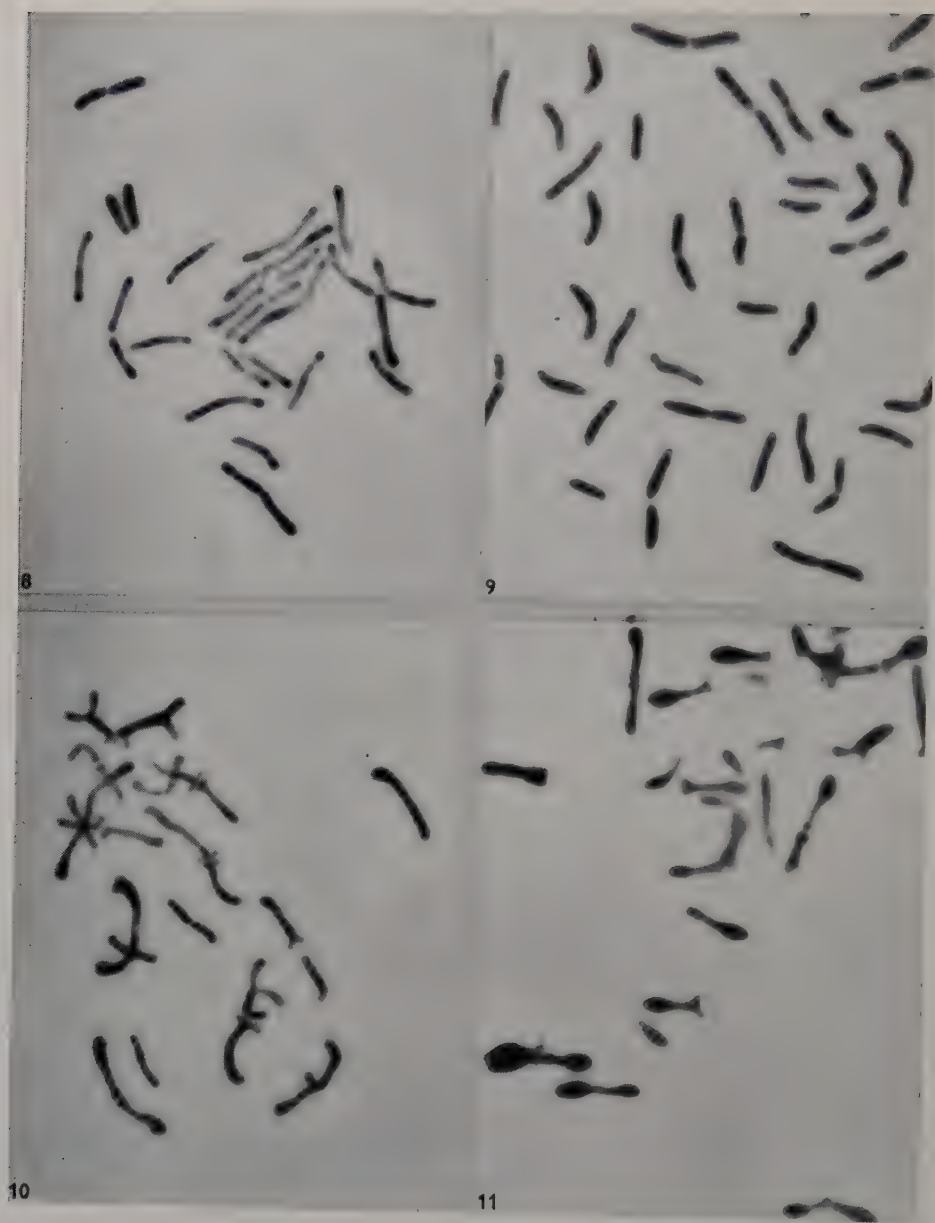
Fig. 8. Strain D₁ (3 days).
Fig. 9. Strain A₆ (1 day).
Fig. 10. Strain C₆ (4 days).
Fig. 11. Strain B₁₁ (1 day).

(Received 27 May 1958)



V. SUNDMAN & K. AF BJÖRKSTEN—GLOBULAR FORMS OF *L. BIFIDUS*. PLATE 1

(Facing p. 496)



V. SUNDMAN & K. AF BJÖRKSTEN—GLOBULAR FORMS OF *L. BIFIDUS*. PLATE 2

The Effect of Actidione and other Antifungal Agents on Nucleic Acid and Protein Synthesis in *Saccharomyces carlsbergensis*

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SUMMARY: The antifungal compound actidione has been found to inhibit both nucleic acid and protein synthesis in *Saccharomyces carlsbergensis*. At the minimum growth inhibitory concentration synthesis of deoxyribonucleic acid and protein is completely inhibited, whereas the synthesis of ribonucleic acid continues for some time after the addition of the antibiotic to the culture. Some other antifungal agents were found to inhibit both nucleic acid and protein synthesis, but in no case was any differential effect obtained.

Investigations on the mode of action of many antibiotics have shown that in a number of cases the biochemical lesion resulting in the bacteriostatic or bacteriocidal effect is located in the sequence of reactions leading to the synthesis of nucleic acid or protein. Fitzgerald, Bernheim & Fitzgerald (1948) reported the inhibition by streptomycin of the synthesis of the enzyme system involved in the adaptive utilization of benzoic acid by *Mycobacterium lacticola*. Gale & Paine (1951) and Gale & Folkes (1953) found that at the minimum growth inhibitory concentration, chloramphenicol, aureomycin and terramycin had little or no effect on respiration, fermentation, or the accumulation of glutamic acid by *Staphylococcus aureus*, but completely inhibited the synthesis of protein by washed suspensions of this organism. In the presence of concentrations of these antibiotics which completely inhibit protein synthesis, there is an increased rate of nucleic acid synthesis. Chloramphenicol has a similar action on nucleic acid and protein synthesis in *Escherichia coli* (Hahn & Wisseman, 1951; Wisseman, Hahn, Hopps & Smadel, 1953; Gros & Gros, 1956). Since the use of antibiotics has provided a useful tool in investigations of the synthesis of nucleic acid and protein in bacteria, a study of the effect of actidione on synthetic processes in the yeast *Saccharomyces carlsbergensis* was initiated. Actidione is produced by *Streptomyces griseus* (Whiffen, Bohonas & Emerson, 1946) has a high degree of activity against many yeasts and fungi (Whiffen, 1948), but has little or no effect on the growth of bacteria or viruses (Hall, Kavanagh & Asheshov, 1951). Actidione was found by Loveless, Spoerl & Weisman (1954) to inhibit both growth and cell multiplication of *Saccharomyces cerevisiae*, but Szilvinyi, Klaus-Hofer & Rauch (1954) were unable to demonstrate any relationship between actidione sensitivity and other physiological properties in *Saccharomyces carlsbergensis*. Although the growth of *Staphylococcus aureus* is insensitive to actidione Creaser (1955) found that a concentration of 3 mg./ml. inhibited by 72% the synthesis of the inducible enzyme β -galactosidase by washed suspensions of this organism.

The present paper describes the effect of actidione on the synthesis of nucleic acid and protein by *Saccharomyces carlsbergensis*. Other antifungal agents were also studied for their effect on nucleic acid and protein synthesis in an attempt to elucidate further the inter-relationship between nucleic acid and protein synthesis. A preliminary report of this work has been given (Kerridge, 1957).

METHODS

Organism and growth medium. The organism used for this study was *Saccharomyces carlsbergensis*, obtained from the Carlsberg laboratory. It has also been called *Saccharomyces mandshuricus*. The organism was maintained by weekly subculture in 2% (w/v) glucose yeast-extract medium (Davies, Falkiner, Wilkinson & Peel, 1951). The organism was grown without shaking in the chemically-defined growth medium of Davies (1956) dispensed in 150 ml. quantities in Roux bottles. The organism was harvested after 16 hr. growth at 30°, washed twice with distilled water and finally resuspended in distilled water to the required concentration. The dry weights of the suspensions were estimated by using a Hilger 'Spekker' absorptiometer previously calibrated for this organism.

Growth inhibition tests. The presence or absence of visible growth was recorded after 16 hr. incubation at 30° using an inoculum of 10^7 organisms/10 ml. of defined medium (Davies, 1956) containing the growth inhibitory agent under test.

Nucleic acid and protein synthesis. For studies on the synthesis of nucleic acid and protein, *Saccharomyces carlsbergensis* was resuspended in the defined medium at a concentration equivalent to 1 mg. dry weight/ml. and aerated at 25°. The antibiotics were added at suitable concentrations to the defined medium. After incubation the organisms were harvested by centrifugation. When logarithmically growing organisms were required, the initial suspension density was adjusted to be equivalent to 0.1 mg. dry weight/ml., the growth followed turbidimetrically and the inhibitor added when the culture had reached a suitable suspension density (*c. equiv.* 0.8 mg. dry weight/ml.). When the incorporation of a ^{14}C -labelled amino acid was to be used as a measure of the synthesis of nucleic acid and protein ($1\text{-}^{14}\text{C}$)-glycine ($0.1\text{ }\mu\text{C./}\mu\text{mole}$) was added to the incubation medium at a final concentration of $0.1\text{ }\mu\text{mole/ml.}$

At the end of the incubation period, samples containing *c. equiv.* 2 mg. dry weight organisms were centrifuged, the organisms washed twice with distilled water and finally resuspended in 5% (w/v) trichloroacetic acid (TCA) and stored at 4° for 2 hr. The precipitate was then centrifuged and washed twice with 5% (w/v) TCA. In experiments where the incorporation of ^{14}C labelled glycine was used as a measure of synthesis, the nucleic acid was extracted with 5% (w/v) TCA at 90°; three 10 min. extractions with 1.0 ml. quantities of the TCA solution being sufficient to remove all the nucleic acid (Gale & Folkes, 1953). The combined supernatant fluids were extracted three times with an equal volume of ether to remove the TCA. The resulting aqueous solution was made up to a known volume with distilled water and a measured sample

transferred to a 2 cm.² polythene planchette. A piece of lens tissue and 0.03 ml. of 0.1 % (w/v) aqueous cetyl trimethylammonium bromide were placed on the planchette to facilitate the spreading of the sample. The planchette contents were dried *in vacuo* and their radioactivity determined by using a Panax Type 100c decadic counter in association with a mica end-window Geiger-Muller tube. Counting was continued for 20 min. or until 1000 counts had been recorded.

The synthesis of nucleic acid was also followed by using the extinction of a hot perchloric acid extract as a measure of the total nucleic acid. The nucleic acid was removed by two 20 min. extractions at 70° with 2.0 ml. 0.5 N-perchloric acid (Ogur & Rosen, 1950). The deoxyribonucleic acid (DNA), content of the perchloric acid extract was measured by the diphenylamine method of Burton (1956).

For the separation of the nucleic acid into the component ribonucleic acid (RNA) and DNA fractions a modification of the Schmidt & Thannhauser (1945) procedure was used. The material precipitated with cold TCA from a sample containing *c. equiv.* 6 mg. dry-weight organisms was resuspended in 1.0 ml. N-NaOH and incubated for 16 hr. at 37°. The alkali was neutralized with 1.0 ml. N-HCl and TCA added to a final concentration of 5 % (w/v). After leaving for 2 hr. at 4° the precipitate was centrifuged down and washed once with 5 % (w/v) TCA. The combined supernatant fluids constituted the RNA fraction. The DNA was extracted from the residue with 5 % (w/v) TCA at 90°. Both RNA and DNA were estimated spectrophotometrically by measuring the extinction of the extract at 260 mμ with a Beckman Model DU spectrophotometer.

The residue after the extraction of the nucleic acid was taken as the protein fraction and was determined: (a) by estimating the nitrogen content by Kjeldahl digestion under the conditions described by Chibnall, Rees & Williams (1943), the ammonia being collected after distillation in a Markham apparatus (Markham, 1942) and determined colorimetrically with Nessler's reagent as described by Johnson (1941); (b) by measuring the incorporation of ¹⁴C labelled glycine into this fraction.

Induction of maltozymase activity in washed suspensions of Saccharomyces carlsbergensis. Induction was carried out aerobically in a system consisting of (% w/v): (NH₄)₂HPO₄, 0.1; (NH₄)H₂PO₄, 0.1; maltose, 1.0; dissolved in McIlvaine buffer pH 5.0 (equivalent to a final concentration 0.05 M-phosphate; McIlvaine, 1921); *S. carlsbergensis* at a final suspension density equiv. 1.0 mg. dry weight/ml.

Estimation of maltozymase activity. The organisms were harvested from the incubation medium by centrifugation, washed twice with distilled water and finally suspended in distilled water to a final concentration equiv. 3.0 mg. dry weight/ml. The maltozymase activity was determined manometrically at 25°, the manometer cups containing 1.5 ml. McIlvaine buffer (pH 5.0) and 1.0 ml. yeast suspension in the main cup, and 0.5 ml. 5 % (w/v) maltose in the side arm; the gas phase was nitrogen.

Chemicals. Kerfoot's Biochemical Reagent sugars were used throughout;

other chemicals were of analytical quality. (1- ^{14}C)-glycine was obtained from the Radiochemical Centre, Amersham. Actidione was obtained from the Upjohn Company, Kalamazoo, Michigan, U.S.A.

RESULTS

Effect of actidione on nucleic acid and protein synthesis

Preliminary investigations. The manometric techniques of Umbreit, Burris & Stauffer (1949) were used to study the effect of actidione on oxygen consumption and anaerobic production of carbon dioxide by *Saccharomyces carlsbergensis* in the presence of glucose. Actidione at concentrations up to 1 mg./ml. inhibited oxygen uptake or carbon dioxide production by not more than 20%. The uptake of glycine and glutamic acid in the presence of glucose into the soluble fraction extractable with cold 5% (w/v) TCA was not inhibited by actidione at a concentration of 0.1 mg./ml. DeMoss & Novelli (1955) demonstrated a soluble protein fraction in micro-organisms which catalyses an L-amino acid dependent exchange of pyrophosphate with ATP; in the presence of excess hydroxylamine the exchange is inhibited and there is a concomitant production of hydroxamic acids. This system was found in *S. carlsbergensis* and actidione at 0.1 mg./ml. had no effect on the production of amino acid hydroxamates when the soluble protein fraction obtained from organisms disrupted in the Hughes's press (Hughes, 1951) was incubated with a complete mixture of amino acids (0.2 mg. of each/ml.) in the presence of 0.6 M-hydroxylamine.

Nucleic acid synthesis. Preliminary studies showed that the addition of ^{14}C -labelled glycine to a culture of *Saccharomyces carlsbergensis* resulted in the irreversible incorporation of ^{14}C in the adenine and guanine of the nucleic acid and that this incorporation could be used as a measure of synthesis of nucleic acid. Figure 1 shows the effect of increasing concentrations of actidione on the incorporation of ^{14}C -labelled glycine into the nucleic acid fraction of *S. carlsbergensis* during a 2 hr. incubation period. Although actidione at the minimum growth inhibitory concentration (0.5–1.0 $\mu\text{g.}/\text{ml.}$) partially inhibited nucleic acid synthesis the inhibition was not complete at concentrations greatly exceeding this. Fractionation of the nucleic acid into RNA and DNA by the method of Schmidt & Thannhauser showed a differential inhibition of incorporation of glycine carbon into the two types of nucleic acid. Over a 2 hr. incubation period with concentrations of actidione greater than 5.0 $\mu\text{g.}/\text{ml.}$ there was complete inhibition of DNA synthesis whereas the synthesis of RNA was not completely inhibited at 100 $\mu\text{g.}$ actidione/ml. (Fig. 2). The residual RNA synthesis in the presence of actidione could account for the degree of inhibition observed when total nucleic acid synthesis was measured, since the RNA:DNA ratio in this yeast is of the order of 20:1.

The above results were obtained by measuring changes occurring after a 2 hr. incubation period and give little indication of any early effects of actidione on nucleic acid synthesis. The rate of incorporation of ^{14}C -labelled glycine into the nucleic acid fraction was therefore examined both in washed suspensions

prepared from 16 hr. cultures and in logarithmically growing cultures of *Saccharomyces carlsbergensis*. In both cases the tracer amino acid and the inhibitor were added simultaneously to the suspension of yeast. Logarithmically growing organisms appeared to be slightly more sensitive to inhibition by actidione, but otherwise the results were similar (Fig. 3), with low concentrations there was little effect on the initial rate of uptake into the nucleic acid

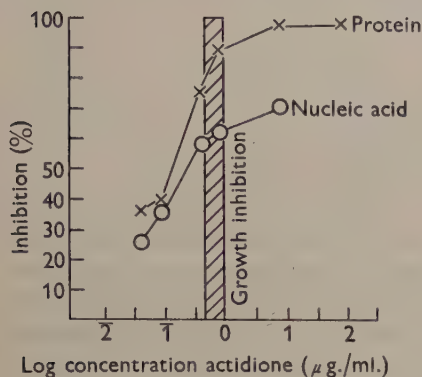


Fig. 1

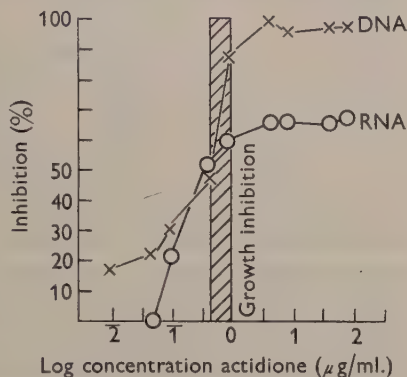


Fig. 2

Fig. 1. Inhibition of nucleic acid and protein synthesis by actidione. *Saccharomyces carlsbergensis* (equiv. 1 mg. dry weight/ml.) was incubated aerobically at 25° in defined medium containing ^{14}C -glycine (0.1 $\mu\text{mole/ml.}$, 0.1 $\mu\text{C}/\mu\text{mole}$) and actidione as required. Organisms were harvested after 2 hr., fractionated by the Schneider technique and uptake of ^{14}C -glycine into the nucleic acid and protein fractions determined.

Fig. 2. Inhibition of nucleic acid synthesis. *Saccharomyces carlsbergensis* (equiv. 1 mg. dry weight/ml.) was incubated aerobically at 25° in defined medium containing ^{14}C glycine and actidione as required. Organisms were harvested after 2 hr., fractionated by the method of Schmidt & Thannhauser and the uptake of ^{14}C -glycine into the RNA and DNA fractions determined.

fraction, but after 30 min. the rate of incorporation was lower than that into the control organisms. At concentrations of actidione greater than 1.0 $\mu\text{g./ml.}$ the initial rate of uptake was decreased and after 60 min. incorporation had almost completely stopped. The results presented so far lead to the conclusion that concentrations of actidione equal to or greater than the minimum growth inhibitory concentration completely inhibit DNA synthesis, whereas the synthesis of RNA continues at the same or at a decreased rate for about 60 min. before it too ceases.

Protein synthesis. Under the experimental conditions used, addition of ^{14}C -labelled glycine to a suspension of *Saccharomyces carlsbergensis* resulted in the irreversible incorporation of ^{14}C in the glycine and serine of the protein fraction; this incorporation was used as a method of following protein synthesis. Actidione at the minimum growth inhibitory concentration almost completely inhibited the incorporation of glycine into the protein fraction (Fig. 1). The adaptive formation of the maltozymase system in *S. carlsbergensis* was similarly inhibited by actidione (Table 1). The effect of less than growth-inhibitory concentrations of actidione on the rate of incorporation

Table 1. *Effect of actidione on the synthesis of the adaptive maltozymase system by Saccharomyces carlsbergensis*

Adaptation system; McIlvaine buffer (pH 5.0), $(\text{NH}_4)_2\text{HPO}_4$ 0.1 % (w/v), $(\text{NH}_4)\text{H}_2\text{PO}_4$ 0.1 % (w/v), actidione as required and organisms added to a final density equiv. 1.0 mg. dry weight/ml. Organisms were harvested after 2 hr. aerobic incubation at 25°, washed twice with distilled water and the maltozymase activity determined manometrically.

Actidione concentration ($\mu\text{g.}/\text{ml.}$)	$Q_{\text{CO}_2}^{\text{N}}$ (maltose) at 25°		Inhibition (%)
	Initial	Final	
Nil	11.2	280	—
0.1	11.2	151	48
1.0	11.2	15	98.5
10	11.2	12	99.7

of ^{14}C -labelled glycine was examined with washed suspensions and with logarithmically growing cultures; the results were similar in both cases (Fig. 4). The inhibition of protein synthesis differed from that of RNA synthesis in that concentrations of actidione greater than 1.0 $\mu\text{g.}/\text{ml.}$ almost completely inhibited protein synthesis and at lower concentrations the time lag before inhibition occurred was less than in the case of nucleic acid.

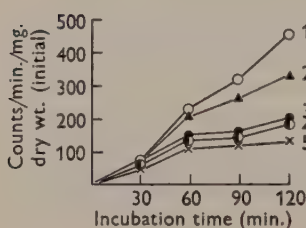


Fig. 3

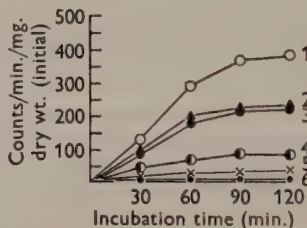


Fig. 4

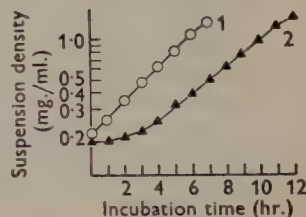


Fig. 5

Fig. 3. Inhibition by actidione of the incorporation of ^{14}C -glycine into the nucleic acid fraction of washed suspensions of *Saccharomyces carlsbergensis*. *S. carlsbergensis* (equiv. 0.6 mg. dry weight/ml.) was incubated aerobically in defined medium containing ^{14}C -glycine (0.1 $\mu\text{mole}/\text{ml.}$, 0.1 $\mu\text{C}/\mu\text{mole}$) and actidione as required. Organisms were harvested after various time intervals and the uptake of ^{14}C -glycine into the nucleic acid fraction determined. Actidione concentration ($\mu\text{g.}/\text{ml.}$): curve 1, control; curve 2, 0.05; curve 3, 0.5; curve 4, 1.0; curve 5, 10.0.

Fig. 4. Inhibition by actidione of the incorporation of ^{14}C -glycine into the protein fraction of washed suspensions of *Saccharomyces carlsbergensis*. *S. carlsbergensis* (equiv. 0.6 mg. dry weight/ml.) was incubated aerobically at 25° in defined medium containing ^{14}C -glycine (0.1 $\mu\text{mole}/\text{ml.}$, 0.1 $\mu\text{C}/\mu\text{mole}$) and actidione as required. Organisms were harvested after various time intervals and the uptake of glycine into the protein fraction determined. Actidione concentration ($\mu\text{g.}/\text{ml.}$): curve 1, control; curve 2, 0.05; curve 3, 0.1; curve 4, 0.5; curve 5, 1.0; curve 6, 10.0.

Fig. 5. Growth of *Saccharomyces carlsbergensis* after preincubation with actidione. *S. carlsbergensis* (equiv. 1.0 mg. dry weight/ml.) was incubated aerobically at 25° in defined medium with or without actidione (1.0 $\mu\text{g.}/\text{ml.}$). Organisms were harvested after 2 hr., washed twice with distilled water, resuspended in defined medium and incubated aerobically at 25°. Growth was followed by estimating the optical density of the suspension at 420 $\text{m}\mu$. \bigcirc — \bigcirc , control culture; \blacktriangle — \blacktriangle , preincubated culture.

*Effect of preincubating with actidione on the subsequent synthesis
of nucleic acid and protein*

Actidione has been shown to be fungistatic, and since it is possible to reverse the inhibition by washing out the antibiotic, the synthesis of nucleic acid and protein after removal of the antibiotic was studied. Organisms which had been first incubated with or without actidione were washed and then re-incubated in fresh medium with ^{14}C -labelled glycine; growth and the incorporation of isotope into the nucleic acid and protein fractions were determined at hourly intervals (Fig. 5). The ratios of the specific activities of the nucleic acid and protein fractions were approximately constant during the period of recovery and subsequent logarithmic growth (control nucleic acid/protein = 0.736 standard deviation = 0.06; preincubated with actidione nucleic acid/protein = 0.740 standard deviation = 0.13). It is therefore unlikely that preferential synthesis of either nucleic acid or protein occurs following release from actidione inhibition.

*The effect of other antifungal agents on nucleic acid and protein synthesis
in *Saccharomyces carlsbergensis**

Actidione at concentrations greater than 1.0 $\mu\text{g./ml.}$ prevents synthesis of DNA and protein and causes inhibition of RNA synthesis after a short time lag. The results suggest that the synthesis of protein is not necessary for the concomitant synthesis of RNA, but throw no light on the inter-relations of DNA and protein synthesis except that the reaction(s) inhibited by actidione is (are) involved in the synthesis of both DNA and protein. Some other growth inhibitors were investigated in an attempt to elucidate further the inter-relations of nucleic acid and protein synthesis in *Saccharomyces carlsbergensis*.

The compounds used were: Crag 341 (2-heptadecyl-2-imidazoline); frequentin, an antibiotic produced by some strains of *Penicillium frequentans* Westling and shown by Curtis, Hemming & Smith (1951) to prevent spore germination in certain fungi and also to possess a high degree of surface activity; gliotoxin (Weindling & Emerson, 1936; Dutcher, Johnson & Bruce, 1945); viridin, produced by *Trichoderma viride* and shown by Brian & McGowan (1945) to inhibit the germination of *Botrytis allii* conidia at 0.005 $\mu\text{g./ml.}$; ethidium bromide. Frequentin, gliotoxin and viridin inhibited nucleic acid and protein synthesis by *Saccharomyces carlsbergensis* to similar extents and inhibition was complete at concentrations equal to or greater than the growth inhibitory concentration (frequentin, 30 $\mu\text{g./ml.}$; gliotoxin, 30 $\mu\text{g./ml.}$; viridin, 0.1 $\mu\text{g./ml.}$).

Crag 341. Inhibition of growth of *Sclerotinia fructicola* by Crag 341 is annulled by guanine (West & Wolf, 1955) and it was therefore of interest as a possible specific inhibitor of nucleic acid synthesis. Figure 6 shows the effect of Crag 341 on nucleic acid and protein synthesis; the addition of purines decreased the degree of inhibition. Aeration of the tubes containing the higher concentrations of Crag 341 caused excessive foaming and subsequent studies demonstrated that the compound was acting as a detergent. Table 2 shows the

effect of Crag 341 on the release of soluble compounds from a suspension of *Saccharomyces carlsbergensis*.

Ethidium bromide. Ethidium bromide is an effective inhibitor of certain protozoa and has been shown to interfere with nucleic acid and protein synthesis in *Strigomonas oncopelti* (Newton, 1957). In view of an apparent structural similarity to actidione its effect on nucleic acid and protein synthesis in yeast was investigated. At concentrations of ethidium bromide less than the minimum growth inhibitory concentration both RNA and DNA synthesis were more sensitive to inhibition than protein synthesis (Fig. 7).

Table 2. *The release of soluble ultraviolet-absorbing material from Saccharomyces carlsbergensis on incubation with Crag 341*

Washed organisms (equiv. 1.0 mg. dry weight/ml.) were incubated with Crag 341. Samples were taken at intervals, organisms removed by centrifugation and the optical density at 260 m μ determined in the Beckman Model DU spectrophotometer.

Inhibitor concn. (μ g./ml.)	Log I_0/I at 260 m μ of the suspending medium					
	0.0	0.3	10.0	30.0	100	1000
Incubation time (min.)						
15	0.046	0.054	0.104	0.215	0.305	0.375
30	0.048	0.053	0.098	0.210	0.288	0.380
60	0.058	0.062	0.096	0.223	0.292	0.400

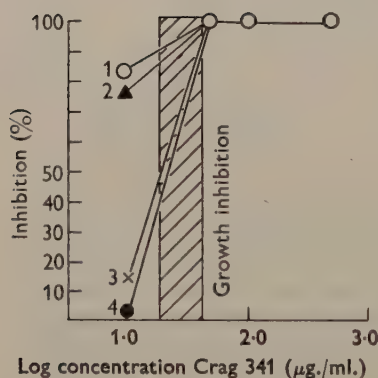


Fig. 6

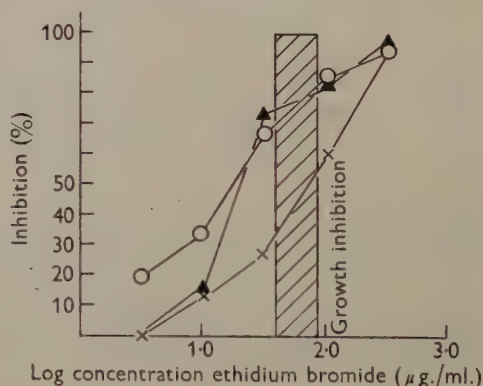


Fig. 7

Fig. 6. Inhibition of protein and nucleic acid synthesis by the fungicide Crag 341. *Saccharomyces carlsbergensis* (equiv. 1.0 mg. dry weight/ml.) was incubated aerobically at 25° in defined growth medium containing 14 C-glycine (0.1 μ mole/ml., 0.1 μ C/ μ mole) and Crag 341 as required. Organisms were harvested after 2 hr., fractionated into the nucleic acid (curves 1 and 4) and protein (curves 2 and 3) fractions determined. Guanine was added to the growth medium in 3 and 4.

Fig. 7. Inhibition of nucleic acid and protein synthesis by ethidium bromide. *Saccharomyces carlsbergensis* (equiv. 1.0 mg. dry weight/ml.) was incubated aerobically at 25° in defined medium to which ethidium bromide had been added as required. Organisms were harvested after 2 hr., fractionated by the method of Ogur & Rosen (1950) and the synthesis of nucleic acid and protein determined. O—O, RNA; ▲—▲, DNA; ×—×, protein.

DISCUSSION

The action of actidione resembles that of the antimicrobial antibiotics chloramphenicol, aureomycin and terramycin in that the primary point of inhibition appears to lie in the sequence of reactions leading to the synthesis of nucleic acid and protein. Actidione differs from these antibiotics in that not only does it inhibit the synthesis of protein at the minimum growth inhibitory concentration but there is also a differential effect on the synthesis of the two types of nucleic acid. At the minimum growth inhibitory concentration the synthesis of DNA and protein is completely blocked, whereas the synthesis of RNA continues at a slower rate for some time after the addition of actidione to the yeast suspension. Actidione has a similar effect on nucleic acid and protein synthesis in *Aspergillus nidulans* (Shepherd, 1958). It has not been possible to locate the exact point of inhibition in the sequence of reactions leading to the synthesis of DNA and protein. Attempts have been made to separate the steps leading to the synthesis of nucleic acid and protein and to study the effect of actidione on individual reactions; but so far no positive results have been obtained.

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The Differential Effect of Temperature on Gas Production by a Coliform Organism

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SUMMARY: The influence of the temperature of incubation on the course of formic acid and gas production by cultures of a coliform organism growing in buffered and unbuffered peptone media was studied. The evidence suggests that at 30° and at 37° the onset of gas production is determined by the concentration of undissociated formic acid in the culture, but that a higher concentration of acid is required to elicit response at 37° than at 30°. Undissociated formic acid is toxic to the organism and it appears that gas is not produced in unbuffered glucose medium at 37° because the environmental conditions do not allow elaboration of hydrogenlyase before inhibitory conditions of pH value and formate concentration are attained.

Coliform bacteria which ferment sugars at 37° with the production of acid and gas are believed to be mainly of intestinal origin and their presence in water is considered indicative of faecal contamination. In routine water analysis occasional coliform strains are encountered which ferment sugars at 37° without the production of gas but which produce acid and gas in the normal way at lower temperatures (Prescott, Winslow & McCrady, 1946). These strains are ordinarily disregarded in the assessment of purity since they are believed to be of non-faecal origin (Stuart, Mickle & Borman, 1940); their anomalous behaviour has received little attention. The gas consists of hydrogen and carbon dioxide and arises by the action of the adaptive hydrogenlyase system on the formic acid produced by carbohydrate breakdown. Wolf, Stickland & Gordon (1954) examined two coliform strains, B6 which produces acid and gas at 30° but only acid at 37°, and C59 which produces acid and gas at 37° but only acid at 44°, when grown in glucose broth. Organisms grown at the higher temperature were devoid of hydrogenlyase but the activity of preformed enzyme was similar at both temperatures; they concluded that enzyme synthesis was inhibited at the higher temperature. Growth of these organisms in glucose broth is noticeably poorer at the higher temperature than at the lower, which suggests that the change in the environmental conditions, which occurs as a consequence of the metabolism of the growing organisms, is more detrimental at the higher temperature. The toxicity of formic acid to coliform organisms increases with decrease in pH value (Gale & Epps, 1942; Dagley, Dawes & Foster, 1953) and since acid is still produced at the higher temperature, inability to synthesize hydrogenlyase may be an important contributory factor in growth inhibition.

Accordingly, the present investigations were concerned with the effect of temperature on the growth and glucose metabolism in peptone media of coliform organism B6, and in particular with its effect on the formic acid/gas system.

METHODS

Media. The medium used for the investigation of the inhibitory effect of formic acid on growth (Table 3) contained: glucose, 0.1 % (w/v); citric acid + KH_2PO_4 buffer, 0.07 M; bacteriological peptone (Oxoid), 1 % (w/v). In all other experiments the medium contained: glucose, 0.5 % (w/v); KH_2PO_4 + Na_2HPO_4 buffer, 0.13 M or Na_2SO_4 , 0.1 M; bacteriological peptone (Oxoid), 1 % (w/v).

Peptone and buffer or sodium sulphate solutions of concentration twice that required in the complete medium were prepared and sterilized separately. Equal volumes of the solutions were mixed in the fermentation vessel (except in the inhibition study where 25 ml. amounts of complete medium were distributed aseptically to 1 oz. screw-capped bottles) and to the mixture was added one twentieth its volume of sterile 10 % or 2 % (w/v) glucose solution.

Formate was included, when needed, by adding an appropriate volume of sterile sodium formate solution of concentration fifty times that required in the medium.

Fermentation procedures. Except for the single experiment where screw-capped bottles were employed, an atmosphere of oxygen-free nitrogen was maintained in the fermentation vessels. In all experiments the vessels were held in thermostatically controlled water baths.

In the initial studies of growth and glucose utilization 250 ml. or 500 ml. conical flasks were used as fermentation vessels. These contained 100 ml. or 200 ml. medium and were fitted with a rubber bung equipped with three short tubes. Two tubes were used for the continuous passage of nitrogen through the flask and the third was wide enough to allow the introduction of an 8 mm. diameter pipette for the removal of samples. When not in use the third tube was closed with a rubber stopper.

For experiments which involved the quantitative determination of gas evolved the constant pressure apparatus shown in Fig. 1 was used. The vessel contained 260 ml. medium. Samples were removed through the side arm. During the preliminary 20 min. gassing of the apparatus and during inoculation or removal of samples, nitrogen was passed continuously through the flask. When gas passage was to be discontinued the stopper was first pushed home and the tap immediately closed. This left a slight positive pressure in the flask.

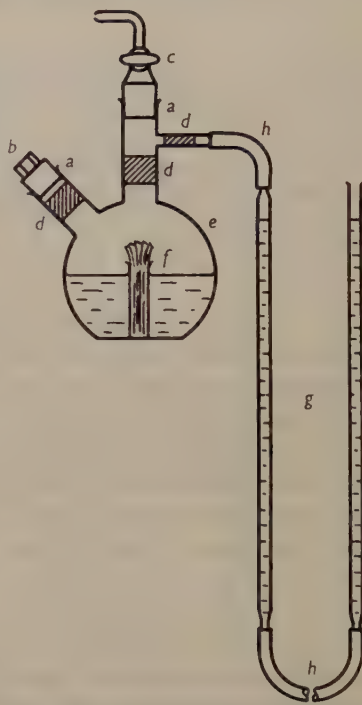


Fig. 1. Constant pressure fermentation vessel. *a*, B24 ground glass joints; *b*, glass stopper; *c*, tap adapter; *d*, cotton-wool plugs; *e*, 500 ml. flask; *f*, centre well containing 4 ml. 40 % (w/v) KOH and 8 × 4 cm. fluted filter-paper; *g*, water manometer (two 50 ml. burettes); *h*, rubber connexions.

The tap was then momentarily opened to relieve the pressure and the manometer level noted. Finally, the right-hand limb of the manometer was lowered to produce a slight reduction of pressure in the flask. Before a determination of hydrogen evolved was made, the vessel was swirled vigorously for about 30 sec. Where the culture was evolving gas there was a rapid initial depression of the manometer level which quickly steadied and was then little affected by continued shaking.

A similar apparatus containing distilled water was placed alongside the fermentation vessel to detect changes in volume caused by changes in atmospheric pressure. The determinations of gas evolved have not been corrected to N.T.P. It is important that the organisms be kept dispersed throughout the medium during the course of a fermentation if reproducible results are to be obtained. To achieve this the fermentation flask was swirled by hand every 20 min. during the phase of active glucose breakdown.

Inoculum. All inoculations were from cultures grown for approximately 18 hr. at the temperature of the main experiment. Where the constant pressure apparatus was used the inoculum was 1 ml. of culture grown in the phosphate-buffered medium (initial pH 6.9). Hydrogenlyase was present in the organisms of these inocula but its activity was much more pronounced in organisms grown at 30° than in organisms grown at 37°.

For other experiments the inoculum medium did not contain glucose but otherwise had the same composition as the medium used in the main experiment and was adjusted to pH 6.9 initially. The volume of inoculum was such that the initial count in the main fermentation was approximately 5 million viable organisms/ml. These organisms did not contain hydrogenlyase.

Estimation of growth. (a) For viable counts, selected dilutions of the culture were plated on nutrient agar (Oxoid) and counted after incubation for 48 hr. at 30°. (b) Opacity measurements of cultures were made with the EEL portable colorimeter, equipped with an orange filter.

Yield and protein content of organisms. The organisms from 200 ml. of culture were recovered by centrifuging, washed once with distilled water, again centrifuged and then suspended in distilled water. The EEL portable colorimeter, equipped with a yellow-green filter, was used for the determination of protein in a sample of suspension by Stickland's (1951) method. The organisms in the remainder were recovered by centrifugation and dried at 100°.

Glucose and formic acid. The determinations were made on samples of culture fluid from which the organisms had been separated by centrifugation. For glucose the culture fluid was diluted 50 fold before analysis when it contained buffer, or 25 fold when it contained sodium sulphate. Glucose was determined by Somogyi's (1945) method.

For formic acid 4 ml. of culture fluid were treated with 4 ml. of 15% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution and 4 ml. 10% (w/v) Ca(OH)_2 suspension and the precipitated glucose and protein sedimented in the centrifuge. Three ml. supernatant fluid mixed with 2 ml. 2 N- H_2SO_4 and 3 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were steam distilled in the Markham still and 50 ml. distillate collected. This was

neutralized to phenolphthalein with 0.01 N-NaOH and then boiled down to approximately 5 ml. The formic acid in this 5 ml. was determined by Hopton's (1953) method.

RESULTS

Influence of buffer capacity of medium

The amount of growth and of glucose consumption by the organism in peptone + sodium sulphate (unbuffered) medium at 30° and 37° are shown in Table 1(a). The adverse effect of the higher temperature is apparent, but at neither temperature had the glucose been completely consumed when multiplication had ceased. A series of qualitative experiments with media containing

Table 1. *Glucose consumption, multiplication and pH change in phosphate-buffered and unbuffered peptone medium at 30° and 37°*

Time (hr.)	Glucose consumed (mg./100 ml.)		pH value		Viable count (in 10 ⁶ /ml.)	
	30°	37°	30°	37°	30°	37°
(a) Unbuffered culture						
0	(479)*	(484)*	6.90	6.90	5	3
3.5	5	9	6.36	5.76	28	36
6	49	33	5.40	5.04	180	28
7	—	43	—	4.82	—	—
8	96	48	5.04	4.72	—	11
10	152	—	4.68	—	342	—
25	262	53	4.30	4.48	202	2
(b) Buffered culture						
0	(462)*	(455)*	6.97	6.95	5	4
4	5	33	6.90	6.84	69	79
5.5	53	115	6.74	6.66	226	278
7.5	197	319	6.55	6.25	—	570
8	272	—	—	—	1270	—
8.5	—	455	—	6.01	—	739
9	462	—	6.31	—	2340	—
24	—	—	6.45	6.38	780	622

* Figures in parentheses are initial concentrations of glucose.

different concentrations of phosphate buffer and of glucose demonstrated that growth could be enhanced at both temperatures when measures were taken to check the decrease in pH value. The results shown in Table 1(b) indicate the improvement which was obtained when 0.1 M-sodium sulphate was replaced by 0.13 M-phosphate buffer. Moreover, in this medium gas was produced in the culture grown at 37° and the organisms showed hydrogenlyase activity when assayed in the Warburg apparatus.

Measurements of the yield and protein composition of organisms obtained under the various cultural conditions (Table 2) when considered with those in Table 1 show that glucose fermentation in buffered medium was accompanied throughout by multiplication. A decrease in the yield of organisms at a temperature higher than the optimum is a common feature of bacterial growth.

In unbuffered medium at 30° fermentation can continue when conditions become unfavourable for cell synthesis, whereas at 37° inhibition of glucose breakdown accompanies inhibition of cell synthesis.

Table 2. Yield and protein content of coliform B6 organisms grown in phosphate-buffered and unbuffered peptone medium at 30° and 37°

Medium and temperature	Time (hr.)	Glucose consumed (mg./100 ml.)	Dry wt. organisms (mg./100 ml.)	Protein/2 mg. dry wt. organism (colorimeter units)
Unbuffered, 30°	7.75	105	19.9	1.06
	24	211	21.4	1.18
Unbuffered, 37°	7	42	6.3	1.18
	24	56	5.7	1.26
Buffered, 30°	9.25	456	70.1	1.08
Buffered, 37°	9	450	56.1	1.10

Quantitative determination of hydrogen evolution and formate production in growing cultures

When organism B6 was grown in unbuffered medium at 37°, formic acid accumulated in the medium (Fig. 2), but no hydrogen had been evolved when glucose breakdown ceased. It is possible that a little free formic acid was formed in the initial stages of the fermentation at 30° but during the phase of brisk hydrogen production the gas-producing system was active enough to

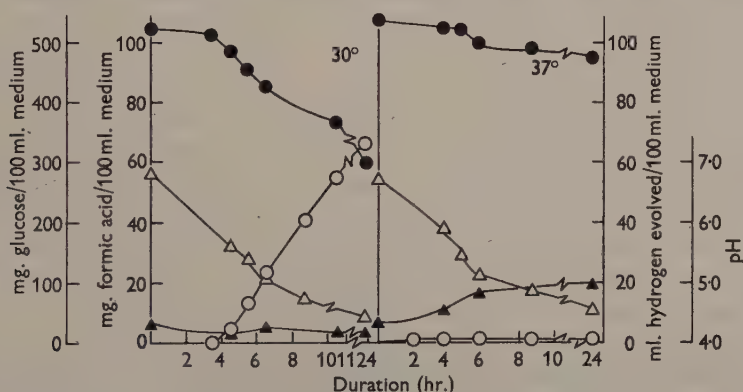


Fig. 2. Dissimilation of glucose in unbuffered peptone medium at 30° and 37°. Initial pH 6.9. Glucose, ●; hydrogen, ○; formic acid, ▲; pH, Δ.

prevent the accumulation of formic acid. Stephenson & Stickland (1932) reported that the optimum pH value for hydrogenlyase in *Escherichia coli* was 7.0; consequently the efficiency of formic acid breakdown at such low pH values seems, at first, surprising. In a later publication, however, Stephenson (1937) mentioned that the optimum pH value of hydrogenlyase changed with the concentration of formate and moved towards the acid side as the amount

of formate was decreased. Studies with washed suspensions of organism B6 have shown that the optimum pH value for hydrogenlyase in this organism is similarly dependent on the formate concentration.

The sequence of events when growth took place in a medium buffered with phosphate initially at pH 6.9 is illustrated in Fig. 3. Hydrogen evolution was only just evident in the 37° culture when the glucose was almost exhausted and when there was abundant formate in the medium. At 30° a trace of

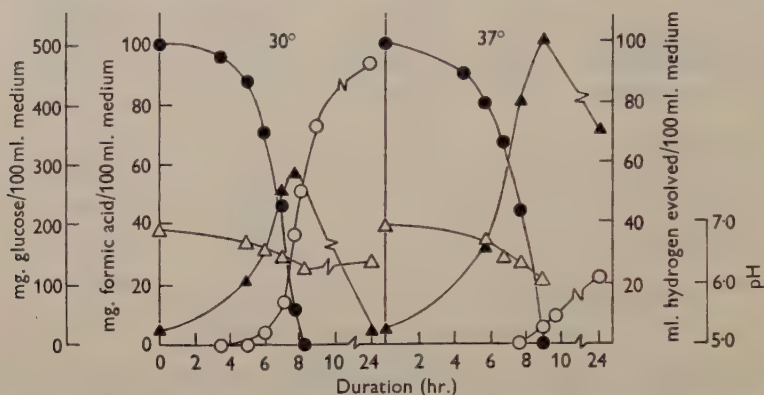


Fig. 3. Dissimilation of glucose in phosphate-buffered peptone medium at 30° and 37°. Initial pH 6.9. Glucose, ●; hydrogen, ○; formic acid, ▲; pH, Δ.

hydrogen had been produced when approximately 60 mg. glucose had been decomposed, at which stage a definite increase in the formate content of the medium was apparent. Subsequently the formate concentration increased still further but hydrogen continued to be evolved during the fermentation of the remainder of the glucose.

Comparison of the behaviour of buffered and unbuffered cultures at 30° suggests that the delay in the response of the gas-producing system (measured in terms of the amount of glucose consumed from the start of fermentation) lessens with decrease in pH value of the medium. This has been shown to be true for other coliform bacteria (Tikka, 1935; Mickelson & Werkman, 1938). That the pH value of the environment exerted a similar influence on the behaviour of organism B6 at both temperatures was made clear when fermentation in medium buffered initially at pH 6.3 was examined (Fig. 4). At both temperatures less glucose breakdown had taken place when hydrogen began to be evolved than in the corresponding cultures grown at the higher stabilized pH range. Here again, however, hydrogen production was not so readily evoked at 37° as at 30°.

It can be inferred from the foregoing experiments that the formate concentration and the pH value of the medium play interrelated parts in eliciting gas production by growing cultures of the organisms used; the evidence points to undissociated formic acid as having a central role in determining the response of the gas-producing system. No gas was produced for some time in the cultures buffered initially at pH 6.9 even though the concentration of formate

had increased and the inoculum organisms contained hydrogenlyase. This may indicate that de-adaptation occurred for a time because the concentration of undissociated acid was kept low and that not until further metabolism had decreased the pH value and increased the formate concentration did re-adaptation, and consequently gas production, begin. This behaviour is being further investigated. The delay in gas production (again measured in terms of

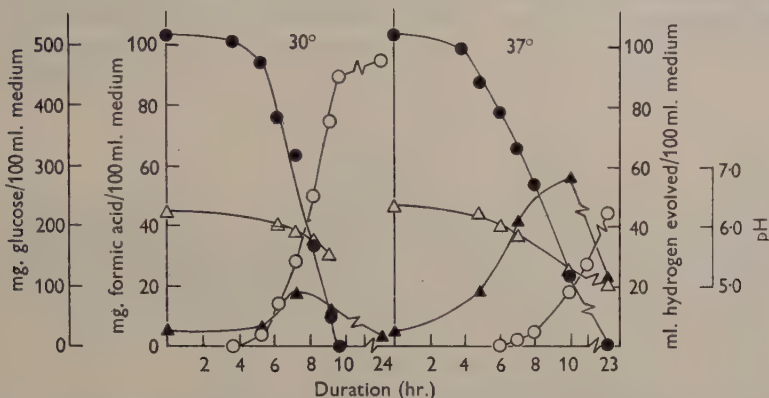


Fig. 4. Dissimilation of glucose in phosphate-buffered peptone medium at 30° and 37°. Initial pH 6.3. Glucose, ●; hydrogen, ○; formic acid, ▲; pH, △.

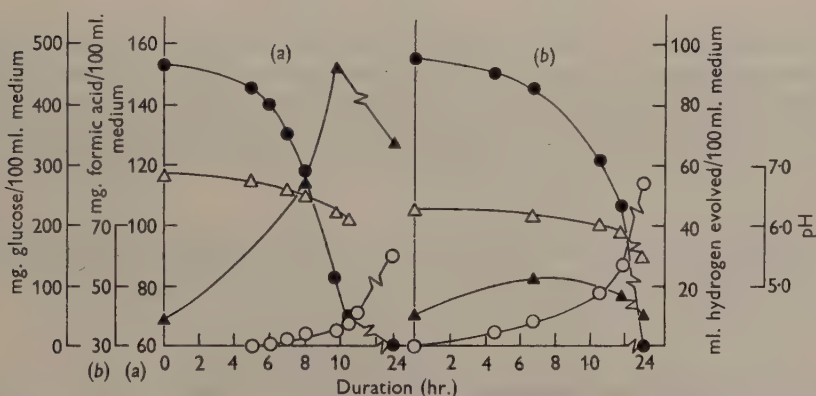


Fig. 5. Dissimilation of glucose in phosphate-buffered peptone medium, containing added formate, at 37°. (a) Initial pH 6.9, initial concentration of formic acid 68 mg./100 ml. (b) Initial pH 6.3, initial concentration of formic acid 40 mg./100 ml. Glucose, ●; hydrogen, ○; formic acid, ▲; pH, △.

glucose consumed) was longer in the 37° culture than in the 30° culture. When growth took place in a medium buffered initially at pH 6.3 this delay was still quite evident at 37°, whereas at 30° it was very short (Fig. 6). This suggests that a higher concentration of undissociated formic acid is required for the initiation of gas production at 37° than at 30°. On this basis it would be expected that inclusion of formate in the medium initially would decrease the delay in gas production and that this would be easier to demonstrate at the

higher temperature. To investigate this the organism was grown in media buffered at pH 6.9 and 6.3 to which formate had been added. The initial concentration was approximately the same as that in the unsupplemented culture at the onset of gas production. Figure 5 shows that gas evolution began at a stage when less glucose had been consumed than in the corresponding culture not containing formate initially. The effect is better illustrated in Fig. 6 where hydrogen evolved is plotted against glucose consumed under the various cultural conditions. The plots have been confined to the results obtained during the period when the progress of fermentation was examined frequently.

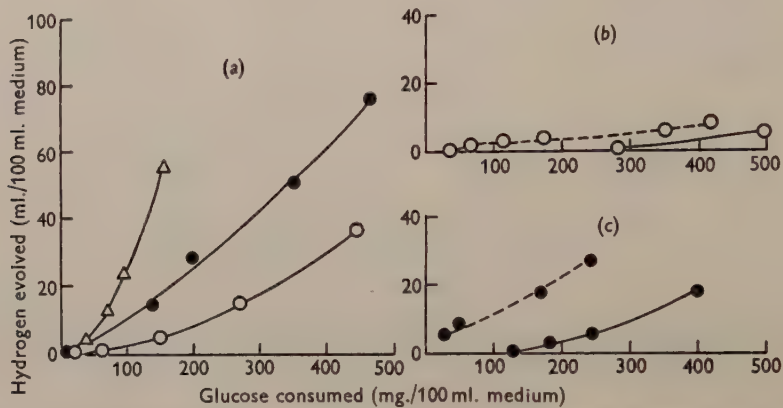


Fig. 6. Plot of hydrogen evolved against glucose consumed during growth under various cultural conditions. (a) 30°: \triangle — \triangle , unbuffered medium; \circ — \circ , buffered medium, initial pH 6.9; \bullet — \bullet , buffered medium, initial pH 6.3. (b) 37°: \circ — \circ , buffered medium, initial pH 6.9; \circ — \circ —, buffered medium containing added formate, initial pH 6.9. (c) 37°: \bullet — \bullet , buffered medium, initial pH 6.3; \bullet — \bullet —, buffered medium containing added formate, initial pH 6.3.

Table 3. *Effect of formic acid on growth of coliform organism B6 in citrate + phosphate-buffered peptone medium at 30° and 37°*

pH value	Initial concn. of formic acid (mg./100 ml.)	Opacity of culture (colorimeter units)			
		7 hr.		24 hr.	
		30°	37°	30°	37°
5.2	0	0.17	0.14	0.65	0.32
	25	0.03	0.03	0.43	0.13
	50	0.02	0.05	0.02	0.05
5.7	0	0.41	0.33	0.69	0.57
	25	0.19	0.15	0.68	0.40
	50	0.08	0.04	0.71	0.30
6.2	0	0.58	0.48	0.69	0.57
	25	0.42	0.42	0.69	0.60
	50	0.33	0.33	0.68	0.57
7.1	0	0.85	0.59	0.84	0.73

The inhibitory effect of formic acid on growth

The effect of formate on growth at 30° and 37° in media buffered at different pH values is shown in Table 3. The lower total yield of organism at the higher temperature was here reflected in the opacity measurements. Growth in the presence of added formate was delayed at both temperatures but only in the medium buffered at the lowest pH value did formate have an effect on the total yield of growth at 30°. At 37° growth at pH 6.2 was unaffected by formate, but at pH 5.7 and 5.2 inhibition increased with increase in formate concentration and at the lowest pH value growth was severely curtailed.

DISCUSSION

The substrate of hydrogenlyase, formic acid, is a product of the metabolism of carbohydrate and the presence of the enzyme in the inoculum organisms is not a prerequisite for the initiation of growth. Thus the situation in the culture at the time of inoculation differs from that where initiation of growth is dependent on the organisms of the inoculum being able to elaborate an adaptive enzyme to attack a substrate which is the sole source of energy in the medium. The evidence of the above work and other observations (Dagley *et al.* 1953; Gale & Epps, 1942) indicate that the situation does become analogous when formic acid becomes potentially toxic, in which case previous formation of hydrogenlyase is obligatory for continued growth.

The experiments with cultures grown in buffered media revealed that the response, or the maintenance of activity, of the gas-producing system was conditioned by the status of the environment as regards pH value and formate concentration; the evidence points to undissociated formic acid as the determinant of activity. The effect of formic acid is qualitatively similar at both temperatures but less acid is required to elicit the response at the lower temperature. The results strongly suggest that the consequent activity is greater at the lower temperature than at the higher. Studies on tetrathionase and penicillinase, two adaptive enzymes which have a similar function to hydrogenlyase in that by their action their toxic substrates are removed, have shown that there is an optimum temperature for their formation (Pollock, 1945; Knox, 1950; Knox & Collard, 1952). With *Bacillus cereus* growth in the presence of penicillin is comparable with growth in its absence provided that the temperature of incubation is not inimical to penicillinase formation (Knox & Collard, 1952). The behaviour of coliform organism B6 with respect to temperature and concentration of undissociated formic acid is obviously similar. At the lower temperature formic acid only affects growth when present in relatively high concentration since the organism can readily form hydrogenlyase. At the higher temperature hydrogenlyase formation is suppressed and although this will be of little consequence where the concentration of undissociated acid is low it will impose a greater limitation where the concentration of acid is higher. Thus it was found that at 37° growth in medium buffered in the region of neutrality was comparable with growth in

the same medium at 30°, but inhibition became greater the lower the pH value and the higher the concentration of formate. The evidence suggests that coliform organism B 6 does not produce gas in unbuffered glucose broth at 37° because the environmental conditions have not allowed elaboration of hydrogenlyase before the pH value has decreased enough to render the accumulated formate toxic.

An appreciable part of this work was carried out in the Department of Agriculture, University of Leeds, and was incorporated in a Ph.D. thesis submitted to the University of Leeds in 1956.

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The Sensitivity of *Vibrio fetus* to Streptomycin and the Emergence of Resistant Mutants

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SUMMARY: Forty-five strains of *Vibrio fetus* were examined for streptomycin sensitivity; in most cases, growth was inhibited by concentrations ranging from 0.5-20 µg./ml. Streptomycin resistance was found in 6 strains. By the replica plating technique it was shown, in the case of 1 strain, that resistance was due to spontaneous mutation which occurred in the absence of streptomycin.

The sensitivity of *Vibrio fetus* to various antibiotics has been reported by Prier (1951), Terpstra & Eisma (1951), Flatla, Braend & Sudboe (1952), Plastring & Easterbrooks (1952), McPherson & Fish (1954), Ryff & Breen (1956) and Mundt (1956). Wide variations were found in the concentrations of both penicillin and streptomycin which inhibited growth, some strains being insensitive to high concentrations of the antibiotics. It is not known whether these strains were naturally insensitive or had acquired a resistance as a result of the widespread use of antibiotics for animal therapy. The development of drug-fastness has been described for many bacterial species; this has recently been reviewed by Schnitzer & Grunberg (1957). In the present work, the sensitivity of *V. fetus* to streptomycin and the emergence of streptomycin-resistant mutants is described.

METHODS

Organisms used. Most of the strains of *Vibrio fetus* studied had been isolated from the vaginal mucus of infected heifers during diagnostic work already reported (Morgan, 1956; Morgan Melrose & Stewart, 1958). The remainder were stock strains received from laboratories in this country and America. All strains were routinely maintained in Brewer's medium and subcultured every 4 weeks.

Antibiotic media. Streptomycin (CaCl₂ complex, Glaxo Laboratories Ltd., Greenford, Middlesex) was used. A stock solution (expressed as µg. of the free base/ml.) was made in distilled water and used fresh. Both liquid and solid media containing antibiotics were used and were made as follows.

Brewer's medium. The required volume of the stock solution of streptomycin was added to 100 ml. volumes of the medium and then dispensed in 7.5 ml. volumes in screw-capped bottles. Control tubes, consisting of Brewer's medium without streptomycin, were also set up and all the tubes were incubated overnight to confirm their sterility before use on the following day.

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Organisms for inocula were grown for 3 days on 'thio blood agar' (see below) in 8 oz. medical flats. The growth was checked for purity and then suspended in 5 ml. nutrient broth. One drop was then added to each dilution of the antibiotic in Brewer's medium and to two control tubes. All tubes were incubated at 37° aerobically and examined for evidence of growth daily for 7 days. In all cases where growth occurred, smears were examined and subcultures made on 'thio blood agar' to check for purity and viability.

Thio blood agar. Nutrient agar containing 0.1 % (w/v) sodium thioglycollate (Morgan, 1957) was melted, cooled to 56° and 10 % (v/v) defibrinated sheep blood added. Streptomycin plates were made by adding the required volume of streptomycin solution to this melted blood agar and pouring into Petri dishes. All plates were dried overnight in the incubator before use.

Plates were inoculated with one drop of a 3-day growth in Brewer's medium of the strains to be tested and each plate was inoculated with 8 different strains. In all cases, control plates made from the same batch of medium but not containing streptomycin were also inoculated. All plates were incubated at 37° in jars containing 90 % (v/v) air + 10 % (v/v) CO₂ and examined after 3 and 7 days of incubation.

Estimation of total number of bacteria. This was done by the method of Miles & Misra (1938), with a pipette calibrated to deliver 0.02 ml./drop. Thio blood agar was used for the estimation of the total number of organisms and thio blood agar containing streptomycin (100, 50, 25 µg./ml.) for the total number of resistant organisms.

Replica plating technique. The technique described by Lederberg & Lederberg (1952) was used with the modifications described by Sneath (1955). Sterile velvet was mounted on a wooden block and two sterile pins were used as markers. Strain Langford was spread on a thio blood agar plate and incubated for 72 hr. at 37° in 90 % (v/v) air + 10 % (v/v) CO₂. The sterile velvet was gently pressed on this plate and then pressed in succession on further plates containing 100 µg. streptomycin/ml. as well as on a control plate without antibiotic. All plates were then incubated at 37° in the air + CO₂ mixture and examined after 3 and 7 days of incubation. By using the markers, the position of the resistant colonies on the original plate (not containing streptomycin) was estimated and these colonies were then subcultured on fresh thio blood agar and incubated. This procedure was repeated until a pure culture of resistant organisms was obtained.

RESULTS

*Sensitivity of *Vibrio fetus* to streptomycin*

Forty-five strains of *Vibrio fetus* were examined by the tube-dilution method in Brewer's medium and by the plate method. In Brewer's medium, the concentration of streptomycin which inhibited growth varied from 1 to 20 µg./ml. and, by the plate method, the range was from 0.5 to 10 µg./ml. In general, the results obtained by the two methods were in fair agreement for any particular strain although differences did occur. With some strains, however,

a few colonies were observed on the plates containing 100, 50, 25, 10 and 5 μg streptomycin/ml., with profuse growth on the control plates and on the plate containing 1 μg . streptomycin/ml. Similarly, in the Brewer's medium, it was found that growth occurred in some of the tubes containing high concentrations of streptomycin after 2 days of incubation; in the control tubes, growth regularly occurred after incubation for 24 hr. In all cases, smears showed that the growth consisted of vibrios, and subcultures were made in fresh Brewer's medium. These strains were re-examined by both methods and the results are given in Table 1. Three strains (Langford; 241; 64/3) showed

Table 1. *The emergence of streptomycin resistance in Vibrio fetus*

Strain	Concentrations of streptomycin ($\mu\text{g}/\text{ml.}$)*							
	500	100	50	25	10	5	2.5	1.0
Plate method								
Langford	3	2	4	5	3	2	++	++
Langford 2	—	—	—	—	—	—	+	++
241	4	8	8	N.O.	8	N.O.	N.O.	++
64/3	—	1	—	1	—	—	—	++
DT74	—	—	—	—	—	—	—	++
1980	—	—	—	—	—	—	—	+
HBC1	—	—	—	—	—	—	—	++
Brewer's medium								
Langford	N.O.	N.O.	(++)	(++)	(++)	(++)	++	++
Langford 2	N.O.	N.O.	(++)	(++)	(++)	(++)	++	++
241	N.O.	N.O.	(++)	—	—	—	—	++
64/3	N.O.	N.O.	(++)	—	—	—	—	++
DT74	N.O.	N.O.	(++)	—	(++)	++	++	++
1980	N.O.	N.O.	(++)	—	(++)	—	—	++
HBC1	—	—	—	—	—	—	—	++

Numbers, thus 3, denote the number of colonies observed after 7 days of incubation.

++ = profuse growth in Brewer's medium or confluent growth on plates.

(++) = (in Brewer's medium) growth only after 2 days.

+ = (on plates) moderate growth.

— = no growth after 7 days.

N.O. = no observation made at this concentration.

streptomycin resistance in liquid and on solid media whereas, with the other three strains (Langford 2; DT74; 1980), resistance was observed only in liquid medium.

The growths on the media containing streptomycin were subcultured into Brewer's medium and these cultures were labelled according to the tube or plate from which they were obtained, e.g. Langford 50 denotes colonies of strain Langford from the plate containing 50 μg . streptomycin/ml. After three subcultures in Brewer's medium, all the resistant cultures so isolated were again examined for streptomycin sensitivity by the plate method. In all cases, profuse growth occurred on all the streptomycin plates including the one with 500 $\mu\text{g}/\text{ml}$. It was also observed that the culture initially isolated from the plate containing 5 $\mu\text{g}/\text{ml}$. was as resistant as one isolated from the plate containing 100 $\mu\text{g}/\text{ml}$., showing that there was no stepwise increase in sensitivity in these experiments.

Experiment to demonstrate the mutational origin of streptomycin resistance

This was done by the replica plating technique on strain Langford. At the first 'cycle' nine colonies were observed on the streptomycin plates. At the subsequent cycles the number of colonies on the streptomycin plates varied from about six to sixty, and this was probably due to the difficulty of picking only the parent resistant colony from the 'original' plates not containing streptomycin. Eventually, however, a resistant culture was obtained which had never been in contact with streptomycin. After numerous subcultures in Brewer's medium as well as 'thio blood agar', profuse growth still occurred in the presence of 500 $\mu\text{g./ml.}$ streptomycin.

The proportion of mutants in strain Langford

The proportion of mutants in strain Langford was estimated by finding the total viable counts on streptomycin plates and on control plates. The results obtained are shown in Table 2. A resistant organism was found once in approximately every 7×10^6 organisms, irrespective of the concentration of streptomycin in the medium used for making the counts.

Table 2. *The proportion of resistant organisms in Vibrio fetus strain Langford on plating 0.02 ml. of culture*

Dilution of culture	Plates containing concentrations of streptomycin ($\mu\text{g./ml.}$)			
	100	50	25	0
	No. of colonies/0.02 ml.			
1/2	1	1	1	∞
1/20	0	0	0	∞
1/200	0	0	0	∞
1/2,000	0	0	0	∞
1/20,000	0	0	0	∞
1/200,000	0	0	0	70, 68, 62 (=67)

Total no. of viable organisms = $67 \times 50 \times 200,000$
 $= 6.7 \times 10^8/\text{ml.}$

No. of resistant organisms = $1 \times 50 \times 2 = 100/\text{ml.}$
 ∞ = profuse growth.

All the streptomycin-resistant strains obtained during this work resembled the parent cultures in producing catalase and not producing hydrogen sulphide and were agglutinated by sera produced against the parent strains.

DISCUSSION

Strains of *Vibrio fetus* resistant to high concentrations of streptomycin were described by Mundt (1956), but it is not known whether these were naturally insensitive strains or not. In the present work, growth of strains of *V. fetus* was inhibited by concentrations of streptomycin from 0.5 to 20 $\mu\text{g./ml.}$, but streptomycin resistance occurred in 6 strains. Three of these strains showed resistant colonies by the plate method and all 6 showed the development of

resistance in Brewer's medium, but growth did not always occur in all the tubes. The replica plating technique was used by Lederberg & Lederberg (1952) in order to demonstrate the spontaneous mutational origin of *Escherichia coli* organisms resistant to streptomycin and to bacteriophage. This technique was used by Goldstein (1954) and Sneath (1955) to demonstrate the mutational origin of drug-resistant organisms. The advantage of the technique is that the indirectly selected populations are not exposed at all to the specific agent. By using this technique, it was possible to obtain a mutant of *V. fetus* which was resistant to 500 µg. streptomycin/ml. without previous contact with the antibiotic.

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Studies of Extracellular and Intracellular Bacterial Deoxyribonucleic Acids

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SUMMARY: Highly polymerized bacterial deoxyribonucleic acids (DNAs) obtained from extracellular and from intracellular locations were analysed. DNAs from two strains (*Staphylococcus aureus* and *S. epidermidis*) were of the type having high adenine and thymine contents; two others (*Alcaligenes faecalis* and *Pseudomonas fluorescens*) had a high content of guanine and cytosine. For each micro-organism the composition of extracellular DNA was essentially the same as that of the intracellular DNA. Conditions associated with the extracellular accumulation of DNA in cultures of 3 strains were investigated. *S. aureus* elaborated a deoxyribonuclease (DNase) which required calcium ion and high pH for activity. In a culture medium of c. pH 6 and insufficient calcium, the DNase was inactive and DNA slime accumulated. In cultures of *P. fluorescens* a specific ribonuclease-sensitive DNase inhibitor protected slime DNA from depolymerization by culture DNase. In cultures of *A. faecalis*, also, both a DNA slime and a low concentration of DNase may occur. This enzyme, detected by studying its action against solutions of calf thymus DNA prepared from dried fibres, was activated by various cations within the pH range of cultures. Unlike other DNases, however, it has little capacity to attack the slime-layer DNA, which presumably is more nearly native.

Highly polymerized deoxyribonucleic acid (DNA) may be present extracellularly in cultures of a variety of bacteria, and observable when a slimy sediment or pellicle accumulates (Smithies & Gibbons, 1955; Catlin, 1956). The polymerized DNA commonly remains associated with the gel-like slime, rather than going into solution in the culture fluid as would be expected of uncomplexed DNA. Elastic recoil may be observed by rotating the sediment into a spiral, which gradually unwinds under its own impetus. Microscopic examination of a wet mount quickly prepared from coiled slime shows twitching streamers of organisms embedded in a relatively transparent matrix.

Polymerized extracellular DNA may be detected even in 6-12 hr. broth cultures by centrifugation and study of the slimy organisms. The origin of this DNA is not obvious, since normal young cultures are not suspected usually of having any significant proportion of lysing organisms. In view of the great differences that may be found between DNAs of various bacterial species (Lee, Wahl & Barbu, 1956), it was thought that a difference in the mechanism of formation of intracellular and extracellular DNAs might be revealed by differences in their base composition. Instead, a striking similarity was found in the proportions of bases composing the DNAs isolated from extracellular and from intracellular locations.

When DNA is responsible for the character of a slime, its viscosity and capacity to form a web-like fibrous precipitate in ethanol are rapidly decreased

by low concentrations of crystalline deoxyribonuclease (DNase) that are without effect on the viability of the organisms. An active bacterial DNase released extracellularly by intact organisms or liberated by lysis, likewise, will depolymerize extracellular DNA. Thus, the absence of slime accumulation does not necessarily indicate absence of production of such DNA.

DNases have been found in the organisms or culture fluids of a number of bacteria, including *Streptococcus pneumoniae* (McCarty & Avery, 1946), *S. pyogenes* (McCarty, 1948) *Staphylococcus aureus* (Cunningham, Catlin & Privat de Garilhe, 1956), *Escherichia coli* (Cohen, 1947), *Serratia marcescens* (Zamenhof, Brawerman & Chargaff, 1952), *Salmonella typhi* (Kay, 1954), *Shigella flexneri* (Masui *et al.* 1956), *Pseudomonas fluorescens* (Catlin, 1956), *Brucella suis* and *B. abortus* (Mauzy, Braun & Whallon, 1955), four species of *Clostridium* (Oakley & Warrack, 1951; Warrack, Bidwell & Oakley, 1951), *Bacillus subtilis* and *Mycobacterium avium* (Masui *et al.* 1956). In addition, DNase action has been detected (unpublished survey) in cultures of many other representatives of Enterobacteriaceae (most strains of *Salmonella*, Ballerup strain of *E. freundii*, *Providencia*, *Proteus*), in *Vibrio metchnikovi*, *B. anthracis*, and in a non-pathogenic *Mycobacterium*.

In cultures of *Staphylococcus aureus*, activity of the extracellular DNase generally prevents accumulation of DNA slime. Certain culture conditions, however, decrease DNase activity sufficiently to allow polymerized DNA to accumulate. Clearly, the generality of occurrence of either extracellular DNA or DNase cannot be determined merely on the basis of presence or absence of DNA slime. Investigations are required of the properties of DNases and conditions associated with accumulation of extracellular DNA. Results of experiments with three unrelated strains are presented in this paper.

METHODS

Materials. Dehydrated culture media (brain+heart infusion and heart infusion broth), proteose-peptone, yeast extract, and agar were obtained from Difco Laboratories, Inc., Detroit, Michigan. Pancreatic DNase (once crystallized), pancreatic ribonuclease (crystalline, salt free) and lysozyme (twice crystallized) were obtained from Worthington Biochemical Corporation, Freehold, N.J.

Analytical methods

DNase activity was measured by a previously described viscometric method (Cunningham *et al.* 1956). Results are expressed as before, except that a unit has been adopted, for convenience, one-eighth as large as that used previously.

Deoxyribose (or DNA) was determined by the diphenylamine reaction (Dische, 1955). Pentose was determined by the method of Webb (1956), and values were expressed as ribonucleic acid (RNA). Phosphorus was determined by the method of King (1932), or preferably (because of the explosive properties of perchloric acid), by that of Fiske & Subbarow (1925). Samples of DNA were dried to constant weight at 70° before determination of phosphorus content. Spectrophotometric measurements (in a Beckman Model

DU spectrophotometer) were carried out on DNA dissolved in 0.1 M-NaCl (Chargaff, 1955).

Before base analyses, ribonucleic acid was in some cases (Table 1) removed by treatment with alkali (Smith & Wyatt, 1951). DNA samples were sealed in tubes with formic acid and a minimum volume of air (Wyatt & Cohen,

Table 1. *Purine and pyrimidine contents of deoxyribonucleic acid preparations*

Source of DNA ...	Mole/100 mole total bases			
	Guanine	Cytosine	Adenine	Thymine
<i>Staphylococcus aureus</i>				
Strain SA-B				
Extracellular-1*	20.2	19.1	32.0	28.7
Extracellular-2*	19.0	19.3	33.8	28.0
Intracellular *	21.0	19.0	30.8	29.2
Strain 209 P (whole cells)†	18.5	19.2	31.0	31.2
<i>Staphylococcus epidermidis</i>				
Extracellular*	18.5	17.5	31.7	32.2
Intracellular†	17.5	17.6	31.0	33.9
<i>Pseudomonas fluorescens</i>				
Strain B/S ₁₀₀₀				
Extracellular‡	34.3	30.4	17.8	17.7
Intracellular‡	33.0	30.8	17.3	19.0
<i>Alcaligenes faecalis</i>				
Extracellular†	31.5	37.6	14.0	16.7
Intracellular*	31.8	36.0	15.9	16.2

* Purification by method described, steps 1-5.

† Alkali-treated.

‡ Ribonuclease-treated.

1953) and heated, within metal shields, in an oil bath (Dow-Corning 550 Fluid) at 170-175° for 75 min. The bases released were determined according to Wyatt (1951), except that the chromatograms were developed in butanol + water + ammonia (Hotchkiss, 1948), and recently published optical values were used (Johnson, 1955; Shugar & Fox, 1952) which, however, differ very little from those of Wyatt.

Bacterial deoxyribonucleases

Slightly purified DNases were prepared from *Alcaligenes faecalis* and from *Pseudomonas fluorescens* by the same general method. Brain + heart infusion (litre quantities in Fernbach flasks) was inoculated with a suspension of organisms from 18-20 hr. infusion agar cultures and was incubated at about 25° with agitation for 5-18 hr. Cultures were harvested by centrifugation and the pooled slimy sediments were washed once or twice in 0.1 M-tris [hydroxymethyl] aminomethane (Sigma Chem. Co.) buffer, pH 7.5, with 0.07 M-NaCl). The organisms were resuspended in this buffer to make a final concentration 25-100 times that in the original culture and were disintegrated by 15 min. exposure in a 10 kc. Raytheon sonic oscillator. Large amounts of nucleic acids and other impurities were present in the supernatant fluids after centrifuga-

tion at 32,700 g for 1 hr. at 3° in a Servall superspeed refrigerated centrifuge. Precipitation in the presence of MnCl_2 (Korkes, del Campillo, Gunsalus & Ochoa, 1951) removed some impurities, together with some of the DNase activity. Precipitates were formed first at pH 6 by addition of HCl, second at pH 8.5 with added NaOH, and lastly at about pH 6.8. Each of the fairly massive precipitates was removed by centrifugation in the cold. In the case of *P. fluorescens*, a considerable amount of the activity removed with the first acid precipitate could be extracted with 0.14 M-NaCl solution. The supernatant fluid from the last centrifugation was dialysed overnight in the cold against 2 l. neutralized 0.001 M-(ethylenedinitrilo)-tetraacetic acid, Eastman (EDTA). Further dialysis was carried out for 24 hr. against six to eight changes of demineralized water or NaCl solution (0.5%, w/v). The fluid after centrifugation was lyophilized to dryness. The resulting fluffy buff-coloured material (2–10 units/mg.) was readily soluble (*A. faecalis*) or moderately soluble (*P. fluorescens*) in distilled water.

DNA preparations

Highly purified calf thymus DNA (prepared by the method of Kay, Simons & Dounce, 1952) was used as reference DNA. Aqueous solutions prepared from the dried fibres were used as control in all viscometric tests of DNase activity. Non-bacterial DNA prepared by a method comparable to that used for bacterial DNA was needed as control in some tests. For this purpose, the spleen of a dog was obtained under anaesthesia and immediately homogenized in cold 0.41 % (w/v) sodium dodecyl sulphate solution by means of a Waring blender. Nucleic acid fibres were obtained by the addition of 2 vol. of ethanol. In a similar manner, crude DNA was obtained from thymus tissue of a freshly slaughtered calf. Purification in both cases was carried out as described for bacterial DNA.

The general method for obtaining the extracellular and intracellular preparations of bacterial DNA and the steps of purification are illustrated by the procedure used with *Staphylococcus aureus*, strain SA-B. Cultures in brain + heart infusion with 4 % (w/v) added NaCl were incubated at 36° for 38 hr. The organisms were harvested by centrifugation (1150 g) and were resuspended in a volume of broth equal to one-thirtieth the volume of the discarded supernatant. NaCl was used in *S. aureus* cultures to inhibit the extracellular DNase but was not used in cultures of the other bacteria. After the latter were harvested, the organisms were resuspended in a solution containing NaCl (0.14 M) and sodium citrate (0.015 M; pH 7.4; 'standard buffer' of Zamenhof, Alexander & Leidy, 1953). The suspension of slimy bacterial sediments was stirred mechanically for 10 min. to obtain a homogeneous mixture. This was divided into two portions.

To obtain extracellular DNA stock sodium dodecyl sulphate (Kay *et al.* 1952) was slowly added to one portion of organisms to give a concentration of 0.2 % (w/v). The mixture was stirred mechanically at room temperature for 2 hr. After this time, with the bacteria which had been suspended in 'standard buffer', solid NaCl was slowly added to give a final concentration

of 0.5 M and stirring was continued for an additional 10 min. The mixture was centrifuged (32,700 g; 1 hr.; 3°) and the sedimented organisms were discarded. The beaker into which the supernatant fluid had been decanted was vigorously rotated by hand in a horizontal plane while 2 vol. of ethanol were slowly added. This ideally resulted in the formation of a single mass of fibres, which soon could be lifted out as a whole. The fluid with its content of amorphous precipitate was poured through a fine stainless-steel wire screen to collect any small isolated fibres. The mass of fibres was drained, washed in 75 % (v/v) ethanol in water, frequently changed until the ethanolic solution was no longer milky, flattened as a sheet against the side of the beaker with a stirring rod to free the mass of excess fluid, and dissolved in 30 ml. of 'standard buffer'. This solution of crude DNA was designated extracellular-1.

The second portion of the suspension of organisms was centrifuged (32,700 g; 1 hr.; 3°) and the supernatant fluid decanted. From this fluid a small amount of fibrous precipitate was obtained on addition of ethanol as described above. The well-washed fibres were dissolved in 'standard buffer' and this fraction was designated extracellular-2. (Most other bacteria did not yield this fraction.) The sedimented organisms were resuspended in a solution containing 0.1 M-NaCl and 0.015 M-MgCl₂. Remaining extracellular DNA was digested at 37° with pancreatic DNase (0.005 mg./ml.). The organisms were centrifuged, washed, and resuspended in 20 ml. of a solution containing 0.1 M-NaCl and 0.1 M-EDTA, and were frozen at -55°. When later thawed, the organisms were lysed by lysozyme (0.5 mg./ml.) neutralized EDTA (final concentration 2 mg./ml.), with NaOH added to give final pH 7.6 (Repaske, 1956). After 6 hr. at 25° with occasional hand stirring, the mixture was very thick and sticky. Addition of 2 vol. of ethanol gave a dense precipitate. In this case, fibres could not be lifted from the ethanolic solution without undue loss. Therefore, the material was centrifuged and the sediment was dissolved with overnight stirring in the cold in a solution containing 1.0 M-NaCl with 0.015 M-sodium citrate. The solution was centrifuged, ethanol was added to the supernatant fluid, and the resulting fibres were drained and dissolved in 'standard buffer'. This crude DNA was designated intracellular fraction.

Each of the fractions, thereafter, was carried through the same steps of purification.

(1) Stock sodium dodecyl sulphate was slowly added to give a final concentration of 0.41 % (w/v) (Kay *et al.* 1952). After rapid mechanical stirring for 3 hr., solid NaCl was added to bring the concentration to M and the stirring was continued for 15 min. The solution was then centrifuged (32,700 g; 1 hr.; 3°) and the supernatant fluid decanted. Addition of 2 vol. of ethanol resulted in the formation of fibres, which were washed as described above, and were dissolved in M-NaCl. The sediment was re-extracted in the same way and fibres were added to those from the first extraction.

(2) A 20 % (w/v) solution of cetyltrimethylammonium bromide was added to a final concentration of 2 %. 'Cetrimide' [Eastman technical, crystallized from 40 % (w/v) solution in 95 % (v/v) ethanol in water by storing overnight at 5°] or 'Cetavlon' [generously furnished by Imperial Chemical

(Pharmaceuticals) Ltd., Manchester] were used with apparently similar results. The solution was stirred for 30–60 min. and 1 vol. of distilled water was slowly added, bringing the concentration of NaCl to 0.5 M (Dutta, Jones & Stacey, 1953). The container was rotated uniformly during addition of the water and for a moment thereafter, while a fibrous precipitate formed. The fibres were lifted (or collected by centrifugation, when necessary) and further purified by two reprecipitations from M-NaCl solution by dilution to 0.5 M with water.

(3) The DNA was redissolved in M-NaCl solution with mechanical stirring at 3°. Step 1 was repeated.

(4) The DNA was dissolved in 0.5 M-NaCl solution and calcium chloride was added to make 0.5 M (McCarty & Avery, 1946; Dutta *et al.* 1953). This was stirred for 1–2 hr. and ethanol was then slowly added to a final concentration of 20 % (v/v). Fibres formed which were lifted, drained, and washed in a solution containing 0.14 M-NaCl, 0.5 M-CaCl₂, and 20 % (v/v) ethanol in water. The drained fibres were dissolved in NaCl (M)+citrate (0.02 M) solution (pH 7.4).

(5) Step 1 was repeated. The DNA fibres were redissolved in NaCl (0.5 M)+citrate (0.02 M) solution (pH 7.4). The preparation was subjected to a final centrifugation (32,700 g; 90 min.; 3°) and to the supernatant fluid two volumes of ethanol were added. The resulting fibres were washed repeatedly in 75 % (v/v) ethanol in water, drained, and dissolved in 0.14 M-NaCl solution.

RESULTS

Analyses of DNA preparations

Staphylococcus aureus (*Micrococcus pyogenes* var. *aureus*). The coagulase-positive strain, SA-B, isolated from clinical material, was used because of the interest attached to a study of the extracellular DNA produced by a strain that also was known to produce high levels of extracellular DNase activity. Base analyses of these preparations are given in Table 1, together with values obtained for comparative purposes from alkali-treated DNA extracted from whole organisms (20 hr. infusion agar cultures) of the well-known penicillin test strain, 209P. Other data follow.

Extracellular-1 had 7.1 % phosphorus. Optical density ratios were 250/260 (0.86), 280/260 (0.53); $\epsilon(P)$ 7000. Extracellular-2 had 6.8 % phosphorus. Optical density ratios were: 250/260 (0.86), 280/260 (0.53); $\epsilon(P)$ 6800. Intracellular DNA had 7.2 % phosphorus. Optical density ratios were: 250/260 (0.87), 280/260 (0.53); $\epsilon(P)$ 6800.

The determination of RNA was prevented by a contaminant that interfered with the colour reaction, possibly a capsular polysaccharide (Webb, 1956) or polysugarphosphate (Zamenhof *et al.* 1953).

Staphylococcus (*Micrococcus*) *epidermidis*. A recently isolated strain of this coagulase-negative micro-organism which produced very little extracellular DNase activity (Weckman & Catlin, 1957) was investigated also. Brain+heart infusion cultures, incubated for 42 hr. at 36°, were harvested and extracellular

DNA was obtained as described (extracellular-1 of strain SA-B). Cellular lysis was more difficult to obtain, however. Concentrations of lysozyme and EDTA were doubled, the pH value raised to 9, and the exposure (at 3°) was continued for 2 weeks. During this time the mixture became very thick and sticky, although some of the organisms may have remained intact. (Sodium dodecyl sulphate, 15% (w/v), did not induce lysis of this strain.) The liberated intracellular DNA was extracted, as described, and RNA was removed by alkali treatment. The extracellular DNA contained 2% RNA and 8.3% phosphorus. Optical density ratios were: 250/260 (0.85), 280/260 (0.56); $\epsilon(P)$ 6380. Base analyses are given in Table 1.

Pseudomonas fluorescens. A strain earlier designated as B (Catlin, 1956) was the source of DNA used in studies of DNase action reported in a later section. From this streptomycin-sensitive parent, a strain was derived for genetic study which was resistant to 1000 μ g. dihydrostreptomycin sulphate/ml.; this was a single-step mutant designated B/S₁₀₀₀. DNA preparations from the mutant strain were obtained from 50 hr. cultures in brain-heart infusion incubated at 25°. Extracellular DNA was obtained by the previously described procedure with 0.2% (w/v) sodium dodecyl sulphate. Many of the organisms retained motility during the entire exposure to detergent, indicating that the procedure caused little or no cellular destruction. Agar plates streaked with loopfuls of the mixture at the beginning and at the end of exposure showed qualitatively similar amounts of bacterial growth. Furthermore, the whole procedure for harvesting extracellular DNA was carried out with organisms which, beforehand, were subjected to DNase, were washed and resuspended in NaCl-citrate solution. Failure to obtain fibres upon addition of ethanol showed that no appreciable release of DNA had been effected by the detergent treatment. Cellular lysis was produced, however, by application of 15% (w/v) aqueous sodium dodecyl sulphate (Zamenhof, Reiner, De Giovanni & Rich, 1956) and intracellular DNA was extracted from the lysate. Both DNA preparations were purified by two exposures to detergent (step 1, above) and to RNase, as follows. To 1 mg./ml. solutions in 0.14 M-NaCl, 0.1 mg./ml. pancreatic RNase was added (Chargaff & Zamenhof, 1948; Butler, Johns, Lucy & Simson, 1956). The mixture was incubated at 25° for 7 hr. with periodic addition of 0.01 N-NaOH to maintain pH 6.5–7.5. Thereafter, 0.02 g./ml. of saline-washed Norit A (Zamenhof & Chargaff, 1951) was added and, after storage at 0° for 1 hr., was removed by centrifugation. The solution, made 2 M with solid NaCl, was dialysed against 2 M-NaCl (Butler *et al.* 1956) at 5° for 5 days with frequent changes of the dialysing fluid. DNA was precipitated with 2 vol. of ethanol. The extracellular DNA preparation contained 10% RNA, 8.1% phosphorus, and optical density ratios were: 250/260 (0.94), 280/260 (0.56). Values for intracellular DNA were: 9% RNA, 8.8% phosphorus, 250/260 (0.93), 280/260 (0.55). Table 1 shows base analyses.

Alcaligenes faecalis. Cultures were incubated at about 25° for 70 hr. (with continuous vigorous agitation during the first day) in a medium composed of proteose-peptone (1%, w/v), yeast extract (0.5%, w/v), with K₂HPO₄ solu-

tion added aseptically to a concentration of 0.001 before inoculation. A crude extracellular-1 DNA preparation was obtained, and treated with alkali (see methods). After enzymic removal of extracellular DNA, organisms from which intracellular DNA was to be extracted were disintegrated in the Raytheon sonic oscillator (as described for enzymes). After centrifugation, the sediment was observed to be a soft pink layer and an underlying white adherent mass. The latter became highly viscous when stirred in 2 M-NaCl solution. Fibres of nucleic acid were precipitated upon addition of 2 vol. of ethanol. This intracellular DNA, purified by steps 1-5 (above), contained 7.8% phosphorus. Optical density ratios were 250/260 (0.94), 280/260 (0.52); $\epsilon(P)$ 7200. Base analyses are shown in Table 1.

Factors responsible for accumulation of extracellular DNA

Staphylococcus aureus produces under certain conditions both slime-layer DNA and extracellular DNase. The DNase is unusual in being activated by calcium, instead of magnesium, having an optimum pH about 8.6, and being heat stable (Cunningham *et al.* 1956). High DNase activity is found extracellularly in brain-heart infusion cultures incubated at about 25° with continuous strong agitation for 40-72 hr. (Weckman & Catlin, 1957). Under these conditions polymerized DNA does not accumulate extracellularly. DNA in low yield has been obtained from the slimy masses of organisms harvested by centrifugation from 12-18 hr. unaerated brain-heart infusion cultures of three different strains of *S. aureus*. Considerably higher yields have been obtained from 36-48 hr. cultures in brain+heart infusion with 0.8-1.0 M-NaCl. This concentration of NaCl inhibited DNase activity; nevertheless, the enzyme was present as shown by tests carried out with dilutions of the supernatant culture fluid in the presence of calcium at pH 8.5.

The presence of DNase was detected also in preparations of slime-layer DNA harvested from *Staphylococcus aureus* cultures. Extracted from the culture environment together with the DNA, the enzyme persisted through two steps of purification with sodium dodecyl sulphate and extraction of the DNA fibres with ethanol, with an intervening step with Cetrimide. One such preparation had 8 units DNase activity/mg. dried fibres; the supernatant medium from which the slimy organisms had been harvested contained 12,000 units/ml. No DNase activity was detected in a companion preparation of intracellular DNA extracted from the same culture. Subsequent steps of purification removed the associated DNase from the DNA.

A solution of DNA with associated DNase remained viscous for prolonged periods. Addition of calcium and pH 8.5 buffer resulted in depolymerization. To investigate the influence of these factors on the accumulation of DNA-containing slime in cultures of *Staphylococcus aureus*, strain SA-B, brain+heart infusion was prepared with supplements as outlined in Table 2. Each of the four media was inoculated in quadruplicate; two sets were agitated vigorously (Weckman & Catlin, 1957) during incubation, while the other two sets were unshaken. In spite of the buffers the pH value of shaken brain+heart infusion cultures tended to increase and the pH value of unshaken cultures tended to

Table 2. *Environmental factors affecting deoxyribonuclease activity and accumulation of DNA-containing slime in cultures of Staphylococcus aureus, strain SA-B**

Initial pH value	Calcium	Time sampled (hr.)	Flasks shaken				Flasks not shaken			
			pH value when sampled	Slime	DNase activity (units/ml.)	Colony-forming units/ml.	pH value when sampled	Slime	DNase activity (units/ml.)	Colony-forming units/ml.
6.0	Not added	18	6.0	trace	64	7.0×10^7	6.0	+	16	1.9×10^7
		36	7.7	0	192,000	5.7×10^8	6.0	++	80	1.2×10^8
6.0	Added	18	5.8	0	10,400	5.3×10^8	5.8	++	24	2.3×10^8
		38	7.7	0	184,000	6.7×10^8	5.8	+++	2,240	1.0×10^9
8.0	Not added	18	7.8	0	94,400	6.0×10^8	7.1†	+++	304	6.1×10^8
		38	8.6	0	234,400	5.0×10^8	7.2	+++	2,800	1.0×10^9
7.8	Added	18	7.6	0	32	1.3×10^8	7.6	0‡	16	5.0×10^5
		38	7.7	0	252,000	7.5×10^8	7.4	0	208	6.0×10^7

* The culture medium was brain + heart infusion (Difco) containing either 0.1 M-sodium succinate (pH 6.0) or 0.005 M-tris (pH 8.0). Where indicated, 3.0 ml. of M-CaCl₂ was added to each 387 ml. portion of medium. Media were dispensed in 100 ml. vol. in 500 ml. Erlenmeyer flasks containing fifteen 3 mm. borosilicate beads and were autoclaved at 121° for 15 min. When cool, media were inoculated with approximately 10⁸ organisms/ml. and were incubated at 28°, with or without agitation.

† After the culture was sampled, NaOH was added, raising the pH to 7.6.

‡ Broth was clear with a non-slimy sediment of calcium phosphate.

decrease during incubation. After incubation for 18 hr. one set of shaken cultures was sampled and immediately returned to the shaker. The duplicate set was allowed to stand for 1–2 hr. to permit aggregation and sedimentation of slime-covered organisms. A slimy sediment was observed only in the pH 6 broth without added calcium. Beginning at 36 hr., the flasks on the shaker were removed, sampled again, and allowed to stand; slime was not observed. During the intervening period of incubation, the two succinate media had become alkaline. An unshaken set of cultures was rotated before sampling at 18 hr. and at 38 hr. to disperse slimy masses of organisms with the aid of the beads. (Microscopic examination, however, showed more aggregation in unshaken than in shaken cultures.) The duplicate set of unshaken cultures was left undisturbed to permit observation of slime (+++ indicating the greatest amount). The morphology and Gram reaction of the cocci were generally typical in cultures having low pH values with added calcium and high pH values without added calcium, both shaken and unshaken. In other cultures the cocci tended to be enlarged, indicating that conditions were unfavourable; also, multiplication was retarded. (Numbers of colony-forming units/ml. were determined from averages of the numbers of colonies developing on triplicate brain + heart infusion agar plates spread with measured samples of a suitable dilution of culture, several dilutions being used in each case.)

Slime did not accumulate in the unshaken culture that had an alkaline pH value and added calcium. Lack of slime could not be attributed merely to insufficient multiplication, as indicated by similar results of a comparable test in which 1.5×10^8 colony-forming units/ml. were present after 44 hr. incubation. Slime accumulated in all other cultures, however, as shown in Table 2. Disappearance of these slimes could be brought about by increasing the pH value to 8 and/or adding calcium (or, as might be expected, by adding pancreatic DNase with magnesium). Shaken cultures apparently had a level of DNase high enough to prevent accumulation of slime, if either pH or calcium ion concentration was nearly optimum.

Pseudomonas fluorescens and *Alcaligenes faecalis* accumulate DNA-containing slime under a variety of culture conditions. Some depolymerizing activity against reference thymus DNA, 1–15 units/ml., was detected in cultures of both strains, occasionally even in the presence of DNA slime. In the case of *P. fluorescens*, a ribonuclease-sensitive DNase inhibitor has previously been shown to be associated with extracellular DNA, protecting it from depolymerization (Catlin, 1956).

Characterization of the DNase of *Alcaligenes faecalis* was required before undertaking a study of the slime-layer DNA of this micro-organism. To obtain a more active source of enzyme, extracts of ultrasonic-disintegrated organisms were examined for DNase activity against reference thymus DNA. Moderate degrees of depolymerizing activity were found. The crude *A. faecalis* enzyme, which had been dialyzed against EDTA and water, was activated by a variety of cations. Listed in order of their decreasing activities as determined in viscometric tests at 37° with thymus DNA (1.0 mg/ml.) and tris buffer (0.05 M; pH 7.5) these were: CaCl_2 , CoCl_2 , MnCl_2 , MgCl_2 (all in final concentrations of

0.0025 M), FeSO_4 (0.00025 M), CuSO_4 and ZnSO_4 (0.0005 M); the activity with zinc was only about 20 % of that with calcium. With CaCl_2 (0.005 M) and imidazole hydrochloride buffers (0.05 M), the *A. faecalis* DNase was active over the whole range of pH values tested (5.2–9.0). Optimum activity was found at pH 8.0–8.2. Activity at pH 6.0 was 40 % and, at pH 9.0, 60 % of maximum. In exploratory tests comparable to those described with *Staphylococcus aureus*, addition of either calcium or cobalt to media did not perceptibly influence accumulation of *A. faecalis* slime as compared with controls without added cation.

Masses of slime-covered cells, with a minimum of non-viscous broth, were aspirated from *Alcaligenes faecalis* cultures. Mixed with an equal quantity of buffer, this material was somewhat viscous and formed a rapidly sedimenting mass or web when a drop was allowed to fall into 90 % (v/v) acetone in water. Enzymic depolymerization of the slime-layer DNA was observed first by loss of viscosity, followed later by loss of web-forming capacity. The depolymerized slime when dropped into acetone immediately dispersed as a fine or flocculent precipitate. This occurred within 5 min. after adding pancreatic DNase (0.01 mg./ml.). However, crude *A. faecalis* DNase, which depolymerized thymus DNA (1 mg./ml.) within 3 hr., had no detectable effect upon the culture slime during 2 days of incubation. This same amount of *A. faecalis* DNase, together with pancreatic ribonuclease (0.1 mg./ml.) depolymerized culture slime in 8 hr. Ribonuclease alone and trypsin (0.5 mg./ml.) alone or mixed with *A. faecalis* DNase were without appreciable effect.

The significance of these findings was investigated in viscometric tests under conditions providing optimum nuclease activities. Activity of *Alcaligenes faecalis* DNase was routinely determined with reference thymus DNA (1 mg./ml.) as substrate. Little activity was shown against other preparations, including DNA from *Pseudomonas fluorescens*, *Staphylococcus aureus*, and dog spleen, as well as from *A. faecalis*. (Inhibition of *P. fluorescens* DNase, on the other hand, was observed only with *P. fluorescens* DNA.). The action of pancreatic ribonuclease had suggested that a ribonucleic-acid inhibitor might be responsible for inactivity of the *A. faecalis* DNase. However, pretreatment of the DNA or the DNase preparations with ribonuclease effected only slight increases in DNase activity. No more than a doubling of activity was produced by concentrations of 10^{-1} – 10^{-3} mg. ribonuclease/ml. An alternative explanation for the slight effect involved possible labilizing action by very small quantities of contaminating DNase, which were detected in this crystalline ribonuclease preparation, in confirmation of a previous observation (Zamenhof, 1957). This possibility was explored with DNA preparations extracted from dog spleen, *A. faecalis*, and *P. fluorescens*. The solution of DNA was incubated with a low concentration of pancreatic DNase, which resulted in a diminution of specific viscosity (5–25 %), but which did not prevent formation of a web-like fibrous precipitate in ethanol. Either directly after pancreatic DNase treatment or after an intervening step involving ethanol precipitation, the DNA was tested with *A. faecalis* DNase. Results comparable to those given in Table 3 were obtained with all three DNA preparations. The susceptibility

to depolymerization by *A. faecalis* DNase of a cautiously prepared, undried (Zamenhof, 1957) DNA was greatly increased by pretreatment with 10^{-7} – 10^{-8} mg. pancreatic DNase/ml. Crude thymus DNase also exerted a comparable labilizing effect. The relative resistance of an intact purified preparation of DNA to depolymerization by this DNase of *A. faecalis* suggests the explanation for the persistence of slime layer DNA. Thus far, a DNase having greater capacity to attack such DNA has not been detected in cultures of *A. faecalis*.

Table 3. *Effect of pretreatment with pancreatic deoxyribonuclease on susceptibility of a deoxyribonucleic acid to the subsequent action of Alcaligenes faecalis deoxyribonuclease*

DNA (1 mg./ml.) solution	Pretreatment with pancreatic DNase (mg./ml.)*	Activity (units/ml.)	
		with added <i>A. faecalis</i> DNase†	without added <i>A. faecalis</i> DNase
Thymus (reference)	0	9.9	—
<i>A. faecalis</i>	0	0.7	—
<i>A. faecalis</i>	5×10^{-8}	9.4	0.6‡
<i>A. faecalis</i>	1×10^{-8}	6.2	<0.1‡

* Reaction at 37° for 90 min. with MgCl_2 (0.025 M) in imidazole (pH 7.3) buffer. DNase activity of mixture with 5×10^{-8} mg./ml. pancreatic DNase was 2.2 units/ml.; reduction of viscosity 24 % after 90 min. reaction. Both pretreatment reactions terminated by addition of 2 vol. of ethanol. DNA precipitated as fibres; washed repeatedly in 95 % (v/v) ethanol in water (20 min.) and acetone (20 min.), dried at 50° for 90 min., and dissolved in original volume of water.

† Reactions at 37° with CaCl_2 (0.005 M) in imidazole pH 8.0 buffer.

‡ Residual activity of pancreatic DNase.

The nature of the inhibition of *Pseudomonas fluorescens* DNase was studied. A preparation of *P. fluorescens* (intracellular) DNA that had been deproteinized with two dodecyl sulphate treatments, but which contained 0.29 mg. RNA/mg. DNA, provided the source of DNase inhibitor. DNA (from dog spleen or from calf thymus) prepared in a manner to avoid dehydration (see methods) was found to be somewhat more susceptible than reference thymus DNA to attack by *P. fluorescens* DNase. Therefore, the DNase activity obtained using undried thymus DNA as substrate was designated 100 %. The DNase preparation, though somewhat purified, also contained some inhibitor (as shown by the inhibitor-destroying effect of ribonuclease; see Table 4), which was introduced into each test in a constant amount. The relationship between extent of enzyme inhibition and percentage of *P. fluorescens* DNA in various mixtures with thymus DNA is shown in Fig. 1. With increasing proportions of inhibitor-containing *P. fluorescens* DNA, the percentage inhibition asymptotically approached a value somewhat below 100 %. The curve resembles that shown for the protein-type inhibitor of yeast DNase (Zamenhof & Chargaff, 1949).

The DNase inhibitor of *Pseudomonas fluorescens* was identified as a ribonucleic acid on the basis of its destruction by ribonuclease. To examine the possibility that the effect may have been due to DNase contaminating the crystalline

RNase and to compare the results obtained using the DNases of *P. fluorescens* and *Alcaligenes faecalis*, viscometric tests were carried out with DNA solutions which had been pretreated with either the RNase (boiled 10 min. to destroy the DNase), or the DNase, of pancreas. The results shown in Table 4 indicate that DNase pretreatment of either *P. fluorescens* DNA or reference thymus DNA had little or no effect on the reaction of *P. fluorescens* DNase,

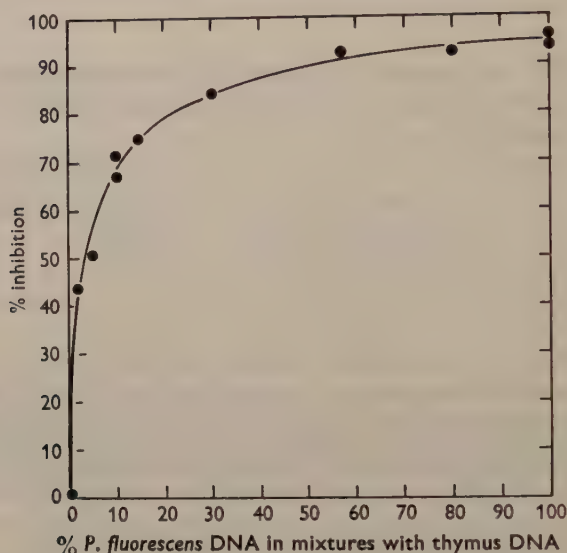


Fig. 1. % Inhibition of *Pseudomonas fluorescens* deoxyribonuclease activity plotted against % *P. fluorescens* DNA in mixtures with thymus DNA. Viscometric tests were carried out with solutions containing 1 mg. DNA/ml., 0.025 M-MgCl₂, imidazole hydrochloride (0.05 M) buffer (pH 7.2), and 1 mg./ml. of a lyophilized, slightly purified DNase preparation obtained from a 7 hr. culture.

added thereafter; the slightly higher rates of reaction against treated DNA as compared with control DNA may be attributed to the continuing action of the pancreatic DNase. Preliminary ribonuclease treatment of DNA markedly increased the reaction rate of the *P. fluorescens* DNase. The increased activity against treated thymus DNA, as compared with untreated control, is due to the destruction of inhibitory RNA which contaminated the preparation of *P. fluorescens* DNase.

The resistance of the preparation of undried *Pseudomonas fluorescens* DNA to attack by *Alcaligenes faecalis* DNase and the susceptibility of reference (dried) thymus DNA are shown in Table 4. The labilizing effect of DNase pretreatment on the more nearly 'native' preparation of DNA is revealed again. Pretreatment of either DNA with boiled RNase did not affect the subsequent rate of attack by the *A. faecalis* DNase.

DISCUSSION

The analytical data obtained for the several extracellular bacterial DNA preparations indicate a general similarity of such material to DNA as found within bacterial and other cells. Representatives appear of both extremes (Chargaff, 1955) of the ratio, adenine + thymine:guanine + cytosine. The low phosphorus content of the detergent-treated preparations previously reported (Catlin, 1956) could be corrected to some extent by additional purification,

Table 4. *Effects of pretreatment with pancreatic deoxyribonuclease or pancreatic ribonuclease on susceptibility of deoxyribonucleic acid preparations to the subsequent action of two bacterial deoxyribonucleases**

	<i>Pseudomonas fluorescens</i> DNA Pretreatment		Calf thymus DNA Pretreatment	
	Pancreatic DNase†	Control	Pancreatic DNase†	Control
	Activity (units/ml.) after addition of DNase from:			
<i>Pseudomonas fluorescens</i>	3.9	2.1	9.8	8.4
<i>Alcaligenes faecalis</i> ‡	7.4	0.1	7.9	8.9

	Pretreatment		Pretreatment	
	Pancreatic ribonuclease§	Control	Pancreatic ribonuclease§	Control
	Activity (units/ml.) after addition of DNase from:			
<i>Pseudomonas fluorescens</i>	15.1	1.9	13.1	8.9
<i>Alcaligenes faecalis</i>	<0.1	<0.1	6.5	6.8

* All incubations at 37°; all concentrations given are those in the actual reaction mixtures. Solutions were kept in an ice bath between pretreatment and subsequent assays. Controls were incubated in exactly the same manner as the enzymically pretreated preparations except for the absence of the crystalline enzyme. To eliminate effect of ageing of the bacterial DNase preparations, data in each horizontal line were obtained by simultaneous assays. One part of bacterial DNase solution was added to seven parts of pretreated DNA solution.

† Enzyme (1.0×10^{-7} mg./ml.) was allowed to act for 60 min. in the presence of 0.1 M-imidazole-HCl pH 7.5 and 0.02 M-MgCl₂, resulting in 25 % reduction in specific viscosity (2.8 units/ml. of DNase activity) of the thymus DNA (1 mg./ml.); and 11 % reduction in specific viscosity (1.3 units/ml.) of the *P. fluorescens* DNA (1 mg./ml.).

‡ Acting in the presence of 0.005 M-CaCl₂.

§ Enzyme (boiled 10 min.) 5×10^{-6} mg./ml., DNA 2 mg./ml., NaCl 0.14 M, pH 7, 90 min. incubation. Buffer-cation solutions were then added in equal volume. The *P. fluorescens* DNase acted in the presence of 0.02 M-MgCl₂ and 0.1 M-imidazole-HCl pH 7.5; *A. faecalis* DNase in 0.005 M-CaCl₂, 0.1 M-imidazole-HCl, pH 8.0.

although the theoretical value was not attained. That part of the remaining impurity is polysaccharide (Jones, 1953) is suggested by the observation that even the best slime-layer DNA preparations show some caramelization on heating with acid. There may also be traces of impurity from the cetyltrimethylammonium bromide (Jones, Marsh & Rizvi, 1957).

The consistent similarity of purine and pyrimidine content (Table 1) of the extracellular and intracellular preparations of DNA from each species

indicates either an ultimate common source of the two or a similarity of mechanisms of synthesis. This result is obtained in spite of variations in preparative methods, with attendant variations in any possible fractionation of the material. Thus, analyses of DNA obtained directly from whole *Staphylococcus aureus*, strain 209P, by a method similar to that used by Lee *et al.* (1956), agree not only with the data of these authors for this species (when calculated by the same method), but also with the base contents of the intracellular and extracellular DNA obtained from strain SA-B by detergent methods.

Similarity of composition of intracellular and extracellular DNA would be expected if the one were liberated by cellular disintegration to give rise to the other. Takahashi & Gibbons (1957) explained in this way the formation of DNA-containing slime by the obligate halophile *Micrococcus halodenitrificans*, cultivated in a peptone medium containing suboptimal concentrations (0.55–0.65 M) of NaCl. Under such conditions, these organisms apparently rupture at a rate which may approach, but does not equal, the rate of multiplication during the logarithmic growth phase. The possibility that one or another disruptive agent may operate in young ordinary cultures of various non-halophilic bacteria requires further investigation. The studies described here have elucidated three circumstances which permit the accumulation of highly polymerized extracellular DNA; however, the reason for its initial appearance remains obscure.

Possible biological consequences of the presence of extracellular DNA are now under investigation. The capacity of highly purified DNA (transforming principle) to convey genetic information is well established, although it cannot be assumed that all DNA has a specific genetic role (Hotchkiss, 1955). Several genetic effects can be conceived. One might be to effect heritable change in competent cells differing in one or more characters. (Indeed, preliminary experiments show that slime-layer DNA from *Neisseria meningitidis* possesses transforming capacity.) The possible occurrence in nature of DNA-conferred genetic change (lysate transformation) has been suggested by Hotchkiss (1951) on the basis of experiments with pneumococci. Addition of penicillin to a culture of a penicillin-sensitive type III strain resulted in lysis of the majority of cocci and liberation of DNA, which transformed penicillin-resistant rough variants to type III capsule production. A second effect of extracellular DNA might be to protect organisms of the population from being transformed, by competing with DNA liberated from variants (see, for example, Hotchkiss, 1954; Lerman & Tolmach, 1957). In this way slime-layer DNA might tend to stabilize the genetic structure of a population. In both these cases, partial depolymerization would result in loss of biological effectiveness. A less direct type of genetic effect, which would require depolymerization of the extracellular DNA, might result from possible selective action of the breakdown products (Braun, Firshein & Whallon, 1957) on elements of a heterogeneous population. Possible non-genetic consequences of the presence of DNA-containing slime, likewise, must be considered, including protection against drying (Smithies & Gibbons, 1955) or against radiation, deleterious

effects of crowding, and action of the polyanionic nucleic acid as a selective barrier to the passage of cations (e.g. see Rogers, 1956).

An unexpected incidental finding of the present study was the specificity of the *Alcaligenes faecalis* DNase for substrate which had been exposed to some form of degradation so mild as to leave the viscosity essentially unchanged. The most susceptible substrate yet used is the reference thymus DNA, which has been stored for several years at room temperature in the form of dry fibres. Cautiously prepared samples of DNA, including one having transforming activity (*Haemophilus influenzae*), are far more resistant to attack by *A. faecalis* DNase. This enzyme may prove a useful tool to indicate slight degradation of DNA structure associated with loss of the 'native state'.

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A Ready Reckoner for the Calculation of Geometric Mean Antibody Titres

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Antibody titrations involve the use of serum dilutions and almost invariably the geometric mean antibody titre calculated for a group of sera gives useful data. The calculation of such geometric mean titres can be both time consuming and tedious, but it can be greatly facilitated by the use of a 'ready reckoner'. Table 1 gives the logarithmic values to base 10 of the reciprocals of serum

Table 1. *Logarithmic values of antibody titres and their multiples*

Antibody titre	Multiples of log ₁₀									
	Log ₁₀	x2	x3	x4	x5	x6	x7	x8	x9	x10
2	0.3010	0.6020	0.9030	1.2040	1.5050	1.8060	2.1070	2.4080	2.7090	3.0100
4	0.6021	1.2042	1.8063	2.4084	3.0105	3.6126	4.2147	4.8168	5.4189	6.0210
6	0.7782	1.5564	2.3346	3.1128	3.8910	4.6692	5.4474	6.2256	7.0038	7.7820
8	0.9031	1.8062	2.7093	3.6124	4.5155	5.4186	6.3217	7.2248	8.1279	9.0310
12	1.0792	2.1584	3.2376	4.3168	5.3960	6.4752	7.5544	8.6336	9.7128	10.7920
16	1.2041	2.4082	3.6123	4.8164	6.0205	7.2246	8.4287	9.6328	10.8369	12.0410
24	1.3802	2.7604	4.1406	5.5208	6.9011	8.2812	9.6614	11.0416	12.4218	13.8020
32	1.5051	3.0102	4.5153	6.0204	7.5255	9.0306	10.5357	12.0408	13.5459	15.0510
48	1.6812	3.3624	5.0436	6.7248	8.4060	10.0872	11.7684	13.4496	15.1308	16.8120
64	1.8062	3.6124	5.4186	7.2248	9.0310	10.8372	12.6434	14.4496	16.2558	18.0620
96	1.9823	3.9646	5.9469	7.9292	9.9115	11.8938	13.8761	15.8584	17.8407	19.8230
128	2.1072	4.2144	6.3216	8.4288	10.5360	12.6432	14.7504	16.8576	18.9648	21.0720
192	2.2833	4.5666	6.8499	9.1332	11.4165	13.6998	15.9831	18.2664	20.5497	22.8330
256	2.4082	4.8164	7.2246	9.6328	12.0410	14.4492	16.8574	19.2656	21.6738	24.0820
384	2.5843	5.1686	7.7529	10.3372	12.9215	15.5058	18.0901	20.6744	23.2587	25.8430
512	2.7093	5.4186	8.1279	10.8372	13.5465	16.2558	18.9651	21.6744	24.3837	27.0930
768	2.8854	5.7708	8.6562	11.5416	14.4270	17.3124	20.1978	23.0832	25.9686	28.8540
1024	3.0103	6.0206	9.0309	12.0412	15.0515	18.0618	21.0721	24.0824	27.0927	30.1030
1536	3.1864	6.3728	9.5592	12.7456	15.9320	19.1184	22.3048	25.4912	28.6776	31.8640
2048	3.3113	6.6226	9.9339	13.2452	16.5565	19.8678	23.1791	26.4904	29.8017	33.1130
3072	3.4874	6.9748	10.4622	13.9496	17.4370	20.9244	24.4118	27.8992	31.3866	34.8740
4096	3.6123	7.2246	10.8369	14.4492	18.0615	21.6738	25.2861	28.8984	32.5107	36.1230

dilutions, together with their multiples. Thus, if in a group of twenty sera, each of two have antibody titres of 8, 32, 128 and 512 and each of four have titres of 64, 256 and 2048, the geometric mean titre for the group may be calculated as shown on p. 541.

No. of sera		Antibody titre	Log ₁₀
2	at	8	1.8062
2	at	32	3.0102
4	at	64	7.2248
2	at	128	4.2144
4	at	256	9.6328
2	at	512	5.4186
4	at	2048	13.2452
Total	20	—	44.5522

$$\text{Log}_{10} \text{ geometric mean titre} = \frac{44.5522}{20} = 2.2276.$$

$$\text{Geometric mean titre} = 169.$$

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The Influence of pH on the Antibacterial Action of Subtilin A

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SUMMARY: The influence of pH value on the antibacterial action of subtilin A has been demonstrated by survivor counts, inhibition of respiration, and pH gradient plates. *Staphylococcus aureus* is more sensitive to subtilin as the pH value increases; *Escherichia coli* is more sensitive as the pH value decreases. The results are analogous to those obtained by other investigators working with cationic detergents, and are consistent with the hypothesis that the basic surface-active antibiotics kill bacteria by the same general mechanism as do the quaternary ammonium germicides. A survey of the growth inhibition of other organisms on pH gradient plates indicates that *Bacillus cereus* behaves like *S. aureus*, while *B. megaterium*, *Corynebacterium poinsettiae*, and *Streptococcus faecalis* behave like *E. coli*.

A pH/mobility curve for subtilin A, obtained by paper electrophoretic studies, indicates no striking changes in the charge of the molecule over the pH range 4-9, although the presence of at least one free α -amino group is indicated by inflexion points in the range of pH 6-7. The isoelectric point of subtilin A at ionic strength 0.1 is approximately 6.7.

The use of sector, square-shaped Petri dishes for pH-gradient plate studies with germicides is described.

The effect of pH value on the germicidal action of cationic detergents is complex: some organisms, including *Staphylococcus aureus*, are more susceptible as the pH value increases, others, including *Escherichia coli*, are more sensitive as the pH value decreases (Salton, 1950). No explanation has been offered to account for this behaviour. Since the discovery by Dubos & Hotchkiss (1942) that tyrocidine shares many of the antibacterial properties of the cationic detergents, a number of surface-active polypeptide antibiotics have been shown to belong to this group of germicides (Newton, 1953; Anderson, Villela, Hansen & Reed, 1946; Sacks, 1952*a*; Colasito, Koffler, Tetrault & Reitz, 1955). However, no extensive study has been made of the influence of pH value on the germicidal action of the surface-active antibiotics, and there has been no demonstration that these pH effects are analogous to those shown for the cationic detergents. Subtilin is a surface-active polypeptide antibiotic, and since it has shown promise as an adjunct in the preservation of both acid (Andersen & Michener, 1950; Wheaton, Burroughs & Hays, 1957) and neutral foods (Godkin & Cathcart, 1953; O'Brien *et al.* 1956) an investigation of the effect of pH value on the germicidal action of this antibiotic was deemed advisable.

METHODS

Subtilin. Subtilin was prepared at this laboratory as previously described (Fevold, Dimick & Klose 1948; Garibaldi & Feeney, 1949). The subtilin thus produced was then fractionated by column chromatography, by the method of

Alderton & Snell (1958). Subtilin A was identified by its migration angle in hanging curtain paper electrophoresis (Sacks & Pence, 1957) and by its high antibacterial activity. It was homogeneous by hanging curtain paper electrophoresis and by the countercurrent distribution methods of Alderton & Snell (to be published). Unfractionated subtilin, lot 326 (70 % potency) was used in the manometric experiments and in some of the paper electrophoretic work.

Turbidimetry. All turbidimetric measurements were carried out with a Coleman model 11 spectrophotometer, at 650 m μ , using 18 mm. test tubes.

Preparation of test organisms. Forty ml. of sterile nutrient broth in a 250 ml. nephelometer flask were inoculated with young cultures of *Staphylococcus aureus* strain H or of *Escherichia coli* strain K 12. The flasks were allowed to incubate overnight at room temperature. They were then placed on a rotary shaker at 35°, and turbidity determined periodically. The organisms were harvested by a gentle centrifugation (2400 g for 3–4 min.) before the culture reached the peak of the log growth phase (optical density = 0.85). It is extremely important to standardize the physiological age of the culture (Sacks, 1952b; Dawson, Lominski & Stern, 1953), and the above procedure makes it possible to obtain cultures of standardized age at a convenient time, just before running an experiment. The organisms were washed in distilled water; then the suspension was made up to o.d. = 0.40. One ml. of this suspension served as the inoculum.

Measurement of bactericidal action of subtilin. A series of test tubes, each containing 6 ml. McIlvaine buffer (diluted 1/3) at graded pH values were placed in a water bath at 36°. One ml. of subtilin A solution (500 p.p.m. for *Escherichia coli*; 200 p.p.m. for *Staphylococcus aureus*) was added to each tube in the series. After temperature equilibration, the tubes were inoculated with the test organism at regular time intervals. After 1 hr. the first tube was plated on nutrient agar, and succeeding tubes were plated at appropriate intervals. Plates were counted after 24 or 48 hr. of incubation.

Manometric experiments. *Staphylococcus aureus* H and *Escherichia coli* 451B were cultivated in medium IIb (Lewis *et al.* 1947) on a shaking machine at 35°. Organisms were harvested before the culture reached the peak of the log growth phase, washed twice in 0.3 % NaCl, and resuspended in this diluent to an optical density of *c.* 0.65. The Warburg vessels contained 1.4 ml. McIlvaine buffer, 0.01 M-glucose, and 0.2 ml. organism suspension in a total volume of 2.0 ml. Subtilin concentrations were 1.0 p.p.m. for *S. aureus*, and 20 p.p.m. for *E. coli*. The subtilin used in these experiments was lot 326. A control vessel from which subtilin was omitted was run at each pH value. Results were calculated by dividing the oxygen uptake of the subtilin-treated organisms (in the time interval of 10–50 min. after tipping the side-arm contents) by the oxygen uptake of the untreated organisms at the same pH values. Temperature was 37°. Control vessels, from which glucose was omitted, showed that there was no appreciable oxidation of the citrate present in the buffer.

pH-gradient plates. A modification of the method previously described

(Sacks, 1956) was used. Square plastic Petri dishes (100 mm.) made it possible to achieve a uniform pH gradient over the entire plate, and also made possible equal streaking distances for all test organisms. The pH gradient was obtained with two 25 ml. layers of Bacto Penassay seed agar (Difco), one supplemented with $M-K_2HPO_4$, the other with $M-KH_2PO_4$ (1/10, v/v). Eugonagar (Baltimore Biological Laboratory, Inc.) was substituted for Penassay seed agar in experiments involving *Corynebacterium poinsettiae*. After the complementary layers had solidified, three pieces of 2 mm. solid glass rod were placed in parallel positions on the surface of the agar in the direction of the gradient, thus creating four compartments in which the pH effect could be demonstrated on the same plate. Two ml. of melted agar containing appropriate amounts of subtilin A were then carefully poured into each compartment. (These subtilin-agar mixtures were prepared slightly in advance and were kept at 45° in 12 mm. screwcap tubes until needed.) After solidification of the surface layer, the test organisms were streaked over each compartment with a sterile swab stick. Suspensions of test organisms were prepared from 24 hr. slopes on Bacto Penassay Base agar, and were made up to an optical density of 0.2–0.4. When the subtilin concentrations were appropriately selected, the plates, after incubation, generally resembled a bar graph, in which pH was the ordinate and subtilin concentration the abscissa. The pH values of uninoculated plates were determined by inserting a glass electrode into the agar.

pH/mobility curve. Paper electrophoresis was employed in these studies because the relative insolubility of subtilin even at low ionic strength renders such a determination difficult by the conventional Tiselius techniques. The low concentrations of polypeptide required for paper electrophoresis made this a satisfactory technique. The subtilin was applied to the paper as unbuffered solutions of 0.35–0.50 % (w/v) concentration. Migrations at various pH values were determined as described previously (Pence, 1953), by techniques based on those of Kunkel & Tiselius (1952). Buffer solutions were used at an ionic strength of 0.1 and consisted of acetate, phosphate, cacodylate (0.02 M-sodium cacodylate + cacodylic acid + 0.08 M-sodium chloride), or barbital (0.01 M-sodium diethyl barbiturate + barbituric acid + 0.09 M-sodium chloride) systems. Whatman 3 MM filter papers were used exclusively. After completion of a run (18 hr., 4.5–5.0 v./cm.) the papers were dried and subsequently stained by the modification of the Rydon & Smith technique developed by Pan & Dutcher (1956). Most of the subtilin used for these experiments was lot 326, which contains about 20 % subtilin B, but since subtilin B has a considerably lower isoelectric point, no difficulties were encountered in interpreting the electrophorograms.

RESULTS

The influence of pH values on the anti-bacterial action of subtilin A was demonstrated by determining plate counts on washed organisms exposed to the antibiotic for 1 hr. in the presence of citrate + phosphate buffers. The results of two typical experiments are shown graphically as survivor/pH curves in

Figs. 1 and 2. Two conclusions emerge from these studies. The effect of pH value is opposite for the two test organisms, and the effect of pH is much more marked for *Escherichia coli* than it is for *Staphylococcus aureus*. Microscopic examination showed no evidence of clumping induced by subtilin.

Manometric experiments were carried out with *Staphylococcus aureus* H and *Escherichia coli* 451B in which the effect of pH value on the inhibition of respiration was determined. The results of two experiments are shown in

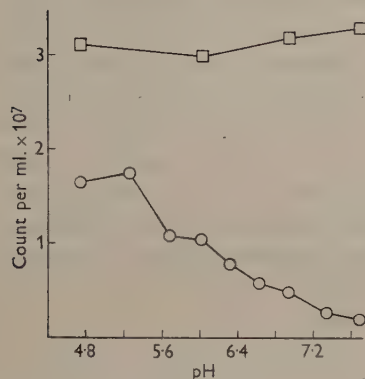


Fig. 1

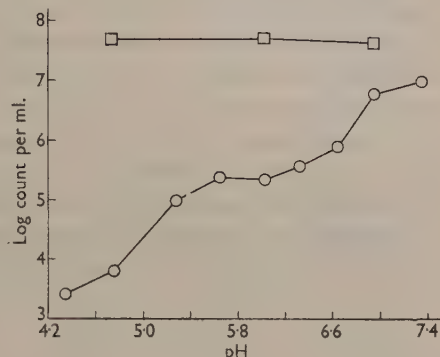


Fig. 2

Fig. 1. pH/survivor curve for *Staphylococcus aureus* H. McIlvaine buffers, diluted 1 to 4. Exposure to subtilin, 60 min., 36°; ○=25 p.p.m. subtilin A; □=control.

Fig. 2. pH/survivor curve for *Escherichia coli* K12. McIlvaine buffers, diluted 1 to 4. Exposure to subtilin, 80 min., 36°; ○=63 p.p.m. subtilin A; □=control.

Fig. 3. The respiration in the controls was not greatly affected by pH value and generally about 200 μ l. oxygen/hr. were consumed, although there was a slight diminution of oxygen uptake (c. 10–30 %) at the highest pH values used. The results obtained with *E. coli* 451B showed a marked increase in sensitivity as the pH value decreased, particularly in the region of pH 7.6–6.4. *S. aureus* H, however, showed no definite change in sensitivity at different pH values in these experiments.

In order to extend and verify these observations, the pH sensitivity to subtilin of several other bacterial species was investigated. It was possible to survey a greater number of species rapidly by use of the pH-gradient agar plate (Sacks, 1956). Many organisms proved unsuitable because they were incapable of growing well throughout the entire pH range of the plate (c. 5.6–7.9). However, six subtilin-sensitive organisms were quickly found which were capable of eugonic growth throughout the entire pH range. For purposes of recording the results photographically, sectorized square Petri dishes were used, making it possible to use several concentrations of subtilin A on the same plate. Results are shown in Pl. 1, in which the opposing effects of pH value on the germicidal action of subtilin A are once more illustrated. *Staphylococcus aureus* H, *S. aureus* S30b, and *Bacillus cereus* v. *terminalis* showed increasing sensitivity to subtilin as the pH value rose. *Corynebacterium poinsettiae*, *B. megaterium* and *Streptococcus faecalis* showed increasing

sensitivity as the pH value decreased. The pH-gradient plates have the disadvantage that bacteriostatic action can also affect the results obtained. Nevertheless, it seems evident that the pH value can affect the germicidal action of subtilin in opposing ways. Some organisms show a slightly increasing sensitivity as the pH value increases, others show a markedly increasing sensitivity as the pH value decreases. The overall picture is analogous to that obtained by Salton (1950) and by Soike, Miller & Elliker (1952) with cationic detergents.

It should be noted that *Escherichia coli* proved extremely resistant to this type of test, growing over the entire pH range, even at very high subtilin concentrations. Perhaps the simplest way to account for this anomaly is to assume the presence of an appreciable number of highly resistant organisms in any clone of *E. coli*.

In considering the effect of pH values on the germicidal action of the synthetic detergents, the charge of the detergent has always been considered to be of paramount importance, the anionic detergents showing completely different pH effects from cationic detergents (Baker, Harrison & Miller 1941 *a*; Gershenfeld & Milanick, 1941; Gershenfeld & Ibsen, 1942).

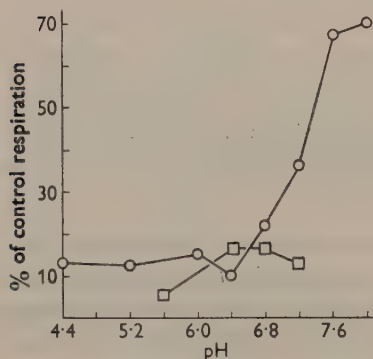


Fig. 3

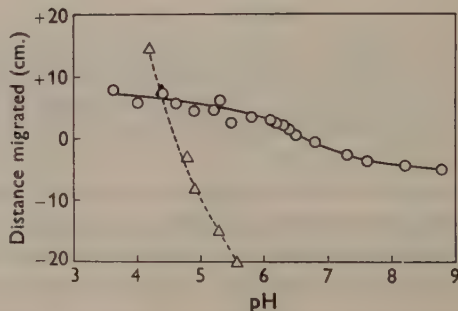


Fig. 4

Fig. 3. Inhibition of O_2 uptake (substrate glucose) by subtilin. McIlvaine buffers, diluted 1 to 1.43. \circ = *Escherichia coli* 451 B; subtilin 20 p.p.m.; \square = *Staphylococcus aureus* H; subtilin 1 p.p.m.; (subtilin, lot 326).

Fig. 4. Effect of pH value on the electrophoretic mobility of subtilin in acetate, cacodylate, phosphate, and barbital buffers at an ionic strength of 0.1 on filter paper, with 4.7 v./cm. for 24 hr. at 25–28°. Results obtained with crystalline bovine serum albumin under similar conditions are included for comparison. \circ = subtilin; \triangle bovine serum albumin.

Earlier studies on high-potency subtilin indicated an excess of free amino groups (Lewis & Snell, 1951) over free carboxyls, and subtilin has generally been considered a basic polypeptide. However, the isoelectric point of subtilin has never been determined, nor have there been any published electrophoretic mobility studies or titration curves for this polypeptide. Because of its obvious relationship to the present study, a pH/mobility curve was drawn from values obtained by horizontal paper electrophoresis (Fig. 4). The curve obtained clearly reveals inflexion points that suggest the presence of at least one α -amino group ($pK=8$) which would be assumed to be part of a lanthio-

nine or β -methyl lanthionine residue (Carson, 1952). Since this article was written, A. Stracher and L. C. Craig indicated in a personal communication to J. C. Lewis of this laboratory that sarcosine very probably furnished the only free amino group in subtilin, other than ϵ -amino groups. The inflexion points are somewhat lower than anticipated, but this very likely results from the effects of bound buffer ions at the ionic strength used (0.1; cf. Velick, 1949; Zittle & Custer, 1957). Considering the excess of amino groups present in subtilin, the relatively low isoelectric point (6.7) is also rather surprising, but this too may probably be explained as the result of bound buffer ions. A similar curve for serum albumin is used to illustrate the low mobility of subtilin at this ionic strength. The heavy adsorption on paper has been previously noted with subtilin (Sacks & Pence, 1957) and with cationic proteins (Monty, Morrison, Alling & Stotz, 1956).

DISCUSSION

This study indicates that the pH value will affect subtilin A activity differently, depending upon the test organism used. In the range pH 4.0–8.0, subtilin A is slightly more effective against *Staphylococcus aureus* and *Bacillus cereus* as the pH rises. It is markedly more effective against *Escherichia coli*, *Corynebacterium poinsettiae*, *Streptococcus faecalis*, and *B. megaterium* as the pH value decreases. These results are in reasonably good agreement with those of Salton (1950) and Soike *et al.* (1952) for cationic detergents, and give added weight to the belief that the surface-active basic polypeptide antibiotics have the same mode of action (Dubos & Hotchkiss, 1942; Newton, 1953). The older theories that cationic detergents are more effective in alkaline solution (Baker *et al.* 1941 a; Gershenfeld & Milanick, 1941; Gershenfeld & Ibsen, 1942) have recently been modified in the light of newer evidence which shows that such pH effects are largely dependent upon the test organism (Salton, 1950; Soike *et al.* 1952). For example, it seems clear that *S. aureus* is more sensitive to the quaternary ammonium compounds in alkaline solution, while the pseudomonads are more sensitive in acid solution. Results reported for *E. coli* indicate increasing sensitivity to cationic surface-active agents as the pH value decreases (Salton, 1950; Soike *et al.* 1952) although this trend is not always clear-cut (Quisno & Foter, 1946; Soike *et al.* 1952).

Adsorption of the cationic detergents increases with increasing pH value regardless of the test organism (Salton, 1950). Thus, it seems likely that some organisms must be able to bind the surface-active agent in a non-toxic way as well as in a toxic way. Indeed, such a concept was enunciated long ago (Baker *et al.* 1941 b). By assuming the presence of at least two different dissociable receptor sites in or near the cytoplasmic membrane, it might be possible to explain the opposing effects of surface-active agents which occur with different microorganisms. If the relative amount of protective binding component varies with different species, such opposing effects may be readily accounted for.

The site of action of the cationic surface-active agents has been a subject of much speculation. Lipoproteins (Dawson *et al.* 1953) phospholipids (Gilby & Few, 1957) polyphosphates (Newton, 1954; Armstrong, 1957) and ribonucleic

acids (Stacey, 1955) have been suggested as the site of action. Any theory purporting to explain the mode of action of the cationic surface-active agents must harmonize with the striking pH effects upon the action of these germicides, and these pH effects may be useful in ruling out certain groups as possible sites of action.

The studies of Carson (1952) and of Lewis & Snell (1951) indicate that subtilin contains some lanthionine or β -methyl lanthionine with free α -amino groups. The pH/mobility curve obtained in this study reveals inflexion points which are a probable reflexion of the presence of these free α -amino groups. The relatively high ionic strengths (0.1) employed account for the apparently low pK indicated. However, the three free ϵ -amino groups of lysine (Carson, 1952; Lewis & Snell, 1951) undoubtedly remain positively charged throughout the range pH 4.0–8.0 used in these experiments, and it seems likely that these ϵ -amino groups play an essential role in the antibacterial action of subtilin (Bichowsky-Slomnicki, Berger, Kurtz & Katchalski, 1956). There is no indication that dissociation of the α -amino groups in subtilin influences its antibacterial action. Somewhat more surprising is the fact that subtilin A exhibits a relatively low isoelectric point for a molecule possessing an excess of free amino groups (Lewis & Snell, 1951). A shift of isoelectric points of proteins as a result of buffer-ion binding has been demonstrated in several instances (cf. Velick, 1949; Zittle & Custer, 1957). At lower ionic strengths effective charge could be much greater, giving greater mobility and a higher isoelectric point.

The use of square Petri dishes to demonstrate the influence of pH value on the antibacterial action of germicides has several advantages over the round plates originally suggested (Sacks, 1956). The square plate provides for a uniform pH gradient in all parallel lines across the plate, it allows an equal streaking distance for all test organisms, it permits the use of divided sections, demonstrated above, and it prevents the slipping of the agar layers in the preparation of the plate. However, the pH-gradient plates are subject to a common defect, viz. they do not distinguish between bactericidal and bacteriostatic effects.

The influence of pH on the action of subtilin as an adjunct in food preservation cannot be predicted on the basis of these results; it will depend on the particular spoilage organisms. Moreover, the use of subtilin to decrease the processing time required to destroy bacterial spores involves several factors not considered here, e.g. the influence of pH value on the adsorption of subtilin by heat-treated spores (Michener, 1955). The work of O'Brien *et al.* (1956) indicated that pH value did not affect the reduction of D values for PA 3679 spores in pea puree.

The influence of pH on the action of impure subtilin has been briefly touched upon in two other publications. Krasnow, Jann & Salle (1953) reported that subtilin exhibited maximum effectiveness in preventing outgrowth of *Clostridium botulinum* spores in two pH ranges, 5.5–6.5, and 8.5–9.0. Housewright, Henry & Berkman (1948) showed that cup plate assays for subtilin had larger inhibition zones in acid media, when *Bacillus cereus* was the test organism.

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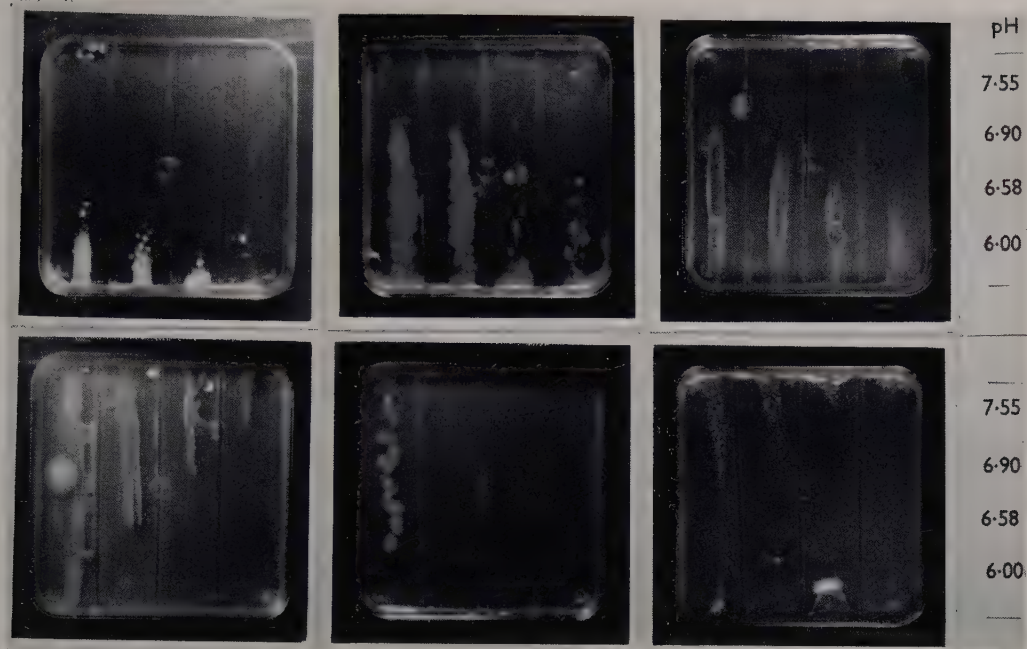
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EXPLANATION OF PLATE 1

pH-gradient plates. Penassay seed agar, potassium phosphate buffers. pH values at right were determined at 24 hr. by inserting a glass electrode into the agar of uninoculated plates. Test organisms, and subtilin A concentration in surface layer are, from left to right. Upper row: *Staphylococcus aureus* H (8, 10, 12, 14 p.p.m.); *Bacillus cereus* var. *terminalis* (50, 60, 70, 80 p.p.m.); *S. aureus* S30b (3, 6, 10, 15 p.p.m.). Lower row: *Corynebacterium poinsettiae* (1, 2, 3, 4 p.p.m.); *B. megaterium* B-938 (1, 2, 3, 4 p.p.m.); *Streptococcus faecalis* (30, 40, 50, 60 p.p.m.).

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L. E. SACKS & J. W. PENCE—pH AND ACTIVITY OF SUBTILIN A. PLATE 1

(Facing p. 550)

An Electro-Taxonomic Survey of Bacteria

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SUMMARY: A taxonomic survey of a wide range of bacteria has been made with the aid of an electrical computing machine. The strains examined fell into five main groups: (1) Gram-positive organisms, including streptococci, micrococci, staphylococci, *Aerococcus*, *Erysipelothrix*, *Listeria* and certain diphtheroids; (2) predominantly Gram-negative organisms, including enterobacteria, pseudomonads *Pasteurella*, *Vibrio*, *Actinobacillus*, *Loefflerella*, *Brucella*, *Neisseria*, *Chromobacterium* and also *Bacillus*; (3) *Corynebacterium diphtheriae*; (4) acid-fast bacilli; (5) spirilla.

Within group 1 were subgroups representing *Streptococcus*, *Micrococcus* and *Staphylococcus*. Within group 2 were subgroups representing: (a) most enterobacteria together with *Pasteurella pestis* and *Vibrio*; (b) *Brucella*, *Neisseria*, *Actinobacillus lignieresii*, *Pasteurella septica* and possibly also *Shigella*; (c) pseudomonads; (d) *Bacillus*; (e) *Chromobacterium violaceum* and (f) *Proteus*.

The taxonomy suggested by this study is compared with that in current use. Temporary working type strains of most of the species are suggested for use in surveys of this kind. The limitations of the method and of using old laboratory records are discussed.

A method of classifying bacteria has been proposed by which an electronic computer is used to indicate the main taxonomic groupings (Sneath, 1957*a*, *b*). The method is based on the Adansonian principles: (1) that every feature is of equal importance in creating taxa; (2) that organisms are classified upon their overall similarity to one another; (3) that overall similarity is the proportion of features possessed in common by two organisms; (4) that divisions between taxa are based on correlated features. These principles follow from the concept that an ideal taxonomy is one containing the greatest content of information in its taxa. An index (S) of overall similarity was proposed, equal to $n_s/(n_s + n_a)$, where n_s was the number of positive features possessed by both of two organisms, and n_a was the number of features possessed by one organism and not the other and *vice versa*. The definition of a feature and the method of analysis were also given.

It was suggested that a survey of a wide range of bacteria might be made, and, since S could be a guide to the taxonomic rank, under certain conditions it could be used to define it. Such a survey might enable a revision of the classification of the Schizomycetes to be made. The present paper is an account of such a survey, but it is emphasized that we do not propose any taxonomic revisions here, since we feel that the results are inadequate for such revision. The purpose of the present paper is to indicate the limitations of using old laboratory records, to suggest strains which might be used as reference strains

in future surveys and to indicate the lines along which more detailed work might profitably be done.

The most important contribution which can be made in a preliminary study like this is to suggest reference strains for future work, since the method requires that if two surveys are to be validly compared then several of the same bacterial strains must be included in both surveys. Ideally these strains would be the type strains of the taxa studied, but in most taxa type strains do not exist, either because they have been lost or because they were never designated. The Judicial Commission of the International Committee on Bacteriological Nomenclature is empowered to establish new type strains where they do not exist, but since the Commission requires that a taxon must be well studied before a neotype is established, for most taxa this will doubtless take many years, if indeed the taxa can be well studied at all without resource to type strains of some sort. We have, therefore, felt it best to break the vicious circle by suggesting certain strains as temporary working type strains for studies of this kind, and some of them may in due course be proposed as neotypes for approval by the Commission. These strains were chosen as typical of the species they represented, and most of them are described in the Collection Catalogue (*National Collection of Type Cultures: Catalogue of Species*, 1958). It should be noted that a type strain need not be typical in every respect (*International Code of Nomenclature of Bacteria and Viruses*, 1958, note to Principle 11), although it must not be 'based upon an abnormality' (Rule 24(h))—the distinction is a nice one. Indeed the more tests used the more difficult it is to find *any* strain which is not atypical in some respect. We have therefore not hesitated to use our own judgement in doubtful instances, although we are aware that some choices will precipitate much argument among experts on the taxa concerned—e.g. *Pasteurella pestis* NCTC 5923 is an avirulent strain chosen for ease and safety of handling. We are much indebted to many colleagues for advice on whether strains are typical, though it has unfortunately not been possible to refer every strain to an acknowledged expert for an opinion.

The importance of type strains is that they determine the application of names whenever taxa are rearranged. Thus, the genus *Bacillus* is by definition composed of those organisms which (in the opinion of an author) belong to the same genus as the type species *Bacillus subtilis*, and hence belong to the same genus as the type strain of *B. subtilis* which is NCTC 3610 (in this instance a neotype, see St John-Brooks & Breed, 1937). If the genus is split into two genera, then *Bacillus* must be retained for the section to which the type belongs. This reference to types extends in practice at any rate up to families—e.g. *Bacillaceae* means 'the *Bacillus* family'. There are various names for types used in botany which indicate different degrees of reliability when a type has not been designated. One nomenclature is given by Ciferri (1957), but his terms apply to strains of the author who named the taxon; this seems to be a doubtful point, for under the Bacteriological Code it would seem more correct for them to apply to strains of the author of the description on which the taxon is based (who need not be the author of the name). The

nomenclature used here is based on the convenient nomenclature of Ainsworth & Bisby (1945).

Holotype. The designated type strain. This takes precedence over any other. If the taxon is based on the description of only one strain this strain would be the holotype by monotypy.

Paratype. Any specimen (other than the holotype) which is specifically stated as being one of the strains on which the original description of the taxon was based (compare Cotype and Isotype).

Cotype. Any specimen of the describing author's collection if he did not designate a holotype strain.

Isotype. Any specimen of the describing author's collection (other than the holotype) when he did designate a holotype strain.

Lectotype. A type taken later for a group for which the author did not designate a type.

Neotype. A type established by the Judicial Commission to replace a designated type which has been lost, or for a group for which a type was not designated. Many neotypes are also lectotypes.

A second reason for publishing this survey is that it is of interest to see what accuracy is attainable with data obtained by standardized methods but read by different observers at different times. The accuracy is clearly less than that of a deliberate comparative study at one time, but may yield useful suggestions for closer study, and large collections of laboratory data must exist which could be used for this purpose.

Validity of the comparisons. In a survey of this kind which uses a wide range of bacteria it is not easy to obtain strictly comparable data. Thus, we cannot make a strictly valid comparison between a strict thermophil and a strict psychrophil, since there is no temperature at which both will grow. We may compare them indirectly by comparing each with a bacterium which is facultatively both thermophilic and psychrophilic. If this is not practicable we must grant the unproved assumption that the growth temperature does not greatly affect the expression of features. This is not an unreasonable assumption (e.g. if we obtained a mesophilic mutant of a thermophilic *Bacillus* we should be unlikely to find that it possessed all the features of a typical *Micrococcus*), but such assumptions must be clearly stated and kept to the minimum. For this reason we have excluded the strictly anaerobic forms (*Clostridium*) from the figures and have added the conclusions in the text only. In the routine practice of the National Collection of Type Cultures the organisms are tested at temperatures near their optimum temperatures, all in the range of 20°–37°, and perforce (without repeating all the work) we have had to assume that this makes little difference to the taxonomic conclusions, though it is not strictly valid. This, and a small number of instances where serum and other adjuvants are necessary to obtain growth in routine peptone or meat extract media, or where the sodium chloride concentration must be decreased, are the only ones where the comparisons are not strictly valid, but there are good reasons for thinking that these factors will not in general greatly influence the features; where there is doubt the data have not been used.

METHODS

Scope of the survey

The National Collection of Type Cultures specializes in bacteria of medical and veterinary interest. This means that some well-known taxa are not represented (e.g. *Rhizobium*), but since bacteria of medical and veterinary importance have as a rule been more thoroughly studied than the others it is not an unsuitable collection for a review of this kind, and in the event of taxonomic revision the nomenclatural types will generally be included in this collection.

In Table 1 are listed the strains used in this survey, with their type status. We have included all designated type strains possessed by the Collection. In Table 2 are shown the relevant parts of the classification used in *Bergey's Manual* (7th edition, 1957), together with the type status of the taxa.

Table 1. *Particulars of strains and of their type status*

The names are those used in the Catalogue of the Collection (*The National Collection of Type Cultures: Catalogue of Species*, 1958), with a few exceptions. The citations of authors will be found in the Catalogue or in *Bergey's Manual*, 7th edition (1957), but they have been given here if the species is not widely known or if confusion might otherwise arise. The name used in *Bergey's Manual* is added in brackets when it is different from that used in the Catalogue. If the species is generally accepted as the type species of the genus the letters T.Sp. have been added.

Number in Fig. 1	NCTC number	Species	Type status of strain
1	5224	<i>Corynebacterium pyogenes</i>	TWT
2	7564	<i>Staphylococcus lactis</i> (Shaw <i>et al.</i> 1951)*	Holotype
3	8163	<i>Erysipelothrix rhusiopathiae</i> T.Sp. (<i>E. insidiosa</i>)	TWT
4	8198	<i>Streptococcus pyogenes</i> , Lancefield group A, T.Sp.	TWT
5	8191	<i>Streptococcus pyogenes</i> , Lancefield group A, T.Sp.	—
6	7760	<i>Streptococcus</i> sp., group M	—
7	8251	<i>Aerococcus viridans</i> (Williams <i>et al.</i> 1953), T.Sp.*	Holotype
8	8029	<i>Streptococcus</i> sp., group O	—
9	8512	<i>Micrococcus luteus</i> T.Sp.	Proposed neotype (Breed, 1952), TWT
10	2416	<i>Chromobacterium viscosum</i> (Grimes, 1927)†*	Holotype
11	2665	<i>Staphylococcus afermentans</i> (Shaw <i>et al.</i> 1951)*	Holotype
12	7973	<i>Listeria monocytogenes</i> T.Sp.	Paratype, TWT
13	8532	<i>Staphylococcus aureus</i> T.Sp.	Proposed neotype (Cowan <i>et al.</i> 1954), TWT
14	7292	<i>Staphylococcus saprophyticus</i> (<i>S. epidermidis</i>)	Proposed neotype (Shaw <i>et al.</i> 1951), TWT
15	8384	<i>Salmonella typhi</i> (<i>S. typhosa</i>)	—
16	8385	<i>Salmonella typhi</i> (<i>S. typhosa</i>)	TWT
17	5735	<i>Salmonella choleraesuis</i> T.Sp.	TWT
18	9001	<i>Escherichia coli</i> T.Sp.	Proposed neotype (Enterobacteriaceae subcommittee) TWT
19	9002	<i>Escherichia coli</i> T.Sp.	—
20	86	<i>Escherichia coli</i> T.Sp.	Cotype
21	5923	<i>Pasteurella pestis</i>	TWT
22	5924	<i>Pasteurella pestis</i>	—
23	9493	<i>Serratia marcescens</i> T.Sp.	—
24	1377	<i>Serratia marcescens</i> T. Sp.	TWT
25	8457	<i>Vibrio</i> sp., El Tor variety	—
26	8021	<i>Vibrio choleraeasiaticae</i> (<i>V. comma</i>) T.Sp.	—

Table 1 (cont.)

Number in Fig. 1	NCTC number	Species	Type status of strain
27	7254	<i>Vibrio cholerae</i> asiaticae (V. comma) T.Sp.	TWT
28	5055	<i>Klebsiella pneumoniae</i> T.Sp.	TWT
29	8016	<i>Loefflerella pseudomallei</i> (<i>Pseudomonas pseudomallei</i>)	—
30	3195	<i>Pasteurella septica</i> (P. multocida)	TWT
31	4976	<i>Actinobacillus lignieresii</i> (A. lignieresii) T.Sp.	TWT
32	4189	<i>Actinobacillus lignieresii</i> (A. lignieresii) T.Sp.	—
33	7470	<i>Brucella melitensis</i> T.Sp.	TWT
34	8223	<i>Brucella melitensis</i> T.Sp.	—
35	8875	<i>Neisseria gonorrhoeae</i> T.Sp.	TWT
36	4887	<i>Shigella dysenteriae</i> , Type 1 (Shiga's bacillus) T.Sp.	TWT
37	6750	<i>Pseudomonas aeruginosa</i> T.Sp.	TWT
38	2000	<i>Pseudomonas aeruginosa</i> T.Sp.	—
39	1691	<i>Loefflerella pseudomallei</i> (<i>Pseudomonas pseudomallei</i>)	—
40	3610	<i>Bacillus subtilis</i> T.Sp., Marburg strain	Neotype (St John-Brooks & Breed, 1937)
41	6276	<i>Bacillus subtilis</i> T.Sp.	—
42	8162	<i>Bacillus pantothenicus</i>	Holotype
43	7464	<i>Bacillus cereus</i>	TWT
44	9757	<i>Chromobacterium violaceum</i> nom. prop. conserv. T.Sp. (Sneath, 1956) (C. janthinum)†	Proposed neotype (Sneath, 1956) TWT
45	9694	<i>Chromobacterium violaceum</i> nom. prop. conserv. T.Sp. (Sneath, 1956) (C. janthinum)†	—
46	9742	<i>Chromobacterium iodinum</i> Davis 1939 (<i>Pseudomonas iodinum</i>)†	Holotype
47	4636	<i>Proteus vulgaris</i> T. Sp.	—
48	235	<i>Proteus morganii</i>	Cotype TWT
49	3708	<i>Loefflerella mallei</i> T.Sp. (<i>Actinobacillus mallei</i>)	TWT
50	9796	<i>Chromobacterium lividum</i> (C. violaceum)†	Proposed neotype (Sneath, 1956) TWT
51	8545	<i>Pseudomonas diminuta</i> (Leifson & Hugh, 1954)*	Paratype
52	3984	<i>Corynebacterium diphtheriae</i> T.Sp. (type gravis)	TWT
53	7429	<i>Corynebacterium diphtheriae</i> T.Sp. (Park 8 strain)	—
54	8151	<i>Mycobacterium phlei</i>	TWT
55	4524	<i>Nocardia farcinica</i> T.Sp.	Cotype
56	8036	<i>Jensenia canicruria</i> (Bisset & Moore, 1950)*	Holotype
57	7762	<i>Spirillum rubrum</i> (<i>Rhodospirillum rubrum</i>) T.Sp.	Cotype
58	9582	<i>Spirillum muncunense</i> (Cayton & Preston, 1955)†*	Holotype
59	8069	<i>Streptobacillus moniliformis</i> T.Sp.	TWT
60	404	<i>Kurthia zopfii</i> T.Sp.	TWT

* Not listed in *Bergey's Manual*, 7th edition (1957).

† Not listed in the Catalogue of the Collection.

‡ The confusion over the name *Chromobacterium violaceum* is because in the past two different species have been given this name: proposals have been made (Sneath, 1956) to regularize the position.

TWT Temporary working type strain used in this study and suggested for use in similar studies, pending the establishment of neotype strains by the International Committee on Bacteriological Nomenclature.

Features

The features and standard techniques are not listed here in view of the preliminary nature of this work, and since it has been concluded (Sneath, 1957b) that any large set of features is likely to yield much the same taxonomic conclusions. These details would of course have to be included if a redescription of the taxa were attempted. However, the scope of the features used was as wide as possible, and these were derived from observations on morphology,

Table 2. *Systematic position of the organisms in the classification of Bergey's Manual, 7th edition (1957)*

Only the more important taxonomic ranks are given, and heavy type indicates that the taxon is the accepted nomenclatural type of the next higher taxon. Names in brackets are those used in Table 1 if they are different from those in *Bergey's Manual*. Species marked with an asterisk are not listed in *Bergey's Manual* and have been inserted in the positions implied by their names or by the authors describing them (not necessarily in the taxonomically correct positions).

Order	Family	Genus	Species	Strain, number in Fig. 1
I	ORDER PSEUDOMONADALES			
	Athiorhodaceae	<i>Rhodospirillum</i>	<i>R. rubrum</i> (<i>Spirillum rubrum</i>)	57
	Pseudomonadaceae	Pseudomonas	<i>P. aeruginosa</i>	37, 38
			<i>P. pseudomallei</i> (<i>Loefflerella pseudomallei</i>)	29, 39
			<i>P. iodinum</i> (<i>Chromobacterium iodinum</i>)	46
			<i>P. diminuta</i> *	51
	Spirillaceae	Spirillum	<i>S. muncunienae</i> *	58
		<i>Vibrio</i>	<i>V. comma</i> (<i>V. cholerae asiaticae</i>)	26, 27
			<i>V. sp.</i> , El Tor vibrio	25
IV	ORDER EUBACTERIALES			
	Rhizobiaceae	<i>Chromobacterium</i>	<i>C. violaceum</i> (<i>C. lividum</i>)	50
			<i>C. janthinum</i> (<i>C. violaceum</i>)	44, 45
			<i>C. viscosum</i> *	10
	Enterobacteriaceae	Escherichia	<i>E. coli</i>	18, 19, 20
		<i>Klebsiella</i>	<i>K. pneumoniae</i>	28
		<i>Serratia</i>	<i>S. marcescens</i>	23, 24
		<i>Proteus</i>	<i>P. vulgaris</i>	47
			<i>P. morgani</i>	48
		<i>Salmonella</i>	<i>S. choleraesuis</i>	17
			<i>S. typhosa</i> (<i>S. typhi</i>)	15, 16
		<i>Shigella</i>	<i>S. dysenteriae</i>	36
	Brucellaceae	<i>Pasteurella</i>	<i>P. multocida</i> (<i>P. septica</i>)	30
			<i>P. pestis</i>	21, 22
		Brucella	<i>B. melitensis</i>	33, 34
		<i>Actinobacillus</i>	<i>A. lignieresii</i>	31, 32
			<i>A. mallei</i> (<i>Loefflerella mallei</i>)	49
	Bacteroidaceae	<i>Streptobacillus</i>	<i>S. moniliformis</i>	59
	Micrococcaceae	Micrococcus	<i>M. luteus</i>	9
		<i>Staphylococcus</i>	<i>S. aureus</i>	13
			<i>S. epidermidis</i> (<i>S. saprophyticus</i>)	14
			<i>S. aerfermentans</i> *	11
			<i>S. lactis</i> *	2
	Neisseriaceae	Neisseria	<i>N. gonorrhoeae</i>	35
	Brevibacteriaceae	<i>Kurtzia</i>	<i>K. zopfii</i>	60
	Lactobacillaceae	<i>Streptococcus</i>	<i>S. pyogenes</i>	4, 5
			<i>S. sp.</i> (group M)	6
			<i>S. sp.</i> (group O)	8
		<i>Aerococcus</i> *	<i>A. viridans</i>	7
	Corynebacteriaceae	Corynebacterium	<i>C. diphtheriae</i>	52, 53
			<i>C. pyogenes</i>	1
		<i>Listeria</i>	<i>L. monocytogenes</i>	12
		<i>Erysipelothrix</i>	<i>E. insidiosa</i> (<i>E. rhusiopathiae</i>)	3
	Bacillaceae	Bacillus	<i>B. subtilis</i>	40, 41
			<i>B. cereus</i>	43
			<i>B. pantothenicus</i>	42
VI	ORDER ACTINOMYCETALES†			
	Actinomycetaceae	<i>Nocardia</i>	<i>N. farcinica</i>	55
		<i>Jensenia</i> *	<i>J. canicruria</i>	56
	Mycobacteriaceae	Mycobacterium	<i>M. phlei</i>	54

† This Order is correctly Order VI not V—see footnote in *Bergey's Manual*, 7th edition, Contents, p. xvi.

staining reactions, colonial form, requirements with respect to oxygen, temperature, certain growth factors, etc., acid production from carbohydrates and other biochemical reactions. The number of features used was 134, but for most strains over half were not recorded. The S values are therefore based on fractions in which the term $(n_s + n_a)$ is usually 30–50. Features based on antigenic structure and on pathogenicity were omitted since the data on these were fragmentary.

Processing of data

The data were coded and analysed on the Elliott 402 Electronic Digital Computer (Elliott Bros., Boreham Wood, Hertfordshire) using the 402 Programme 'Bacterial Classification Mark I'. The results are shown in the diagram (Fig. 1) in which the S values have been shaded; this is accurate enough for the present purpose and is easier to see than entries in figures.

The data on the strains were fed into the computing machine in a random order as a check upon the sorting procedure (steps 5–8 in Sneath 1957*b*, p. 209). This was only partly successful. Although most of the groups were correctly formed, the order in which these groups were placed was not wholly satisfactory, largely because most of the groups fused together in a very limited range of highest intergroup S values around 70 %. Also, several aberrant strains belonging to the main groups were placed at the bottom of the table. On preparing a shaded diagram similar to Fig. 1, it was clear that some rearrangement was necessary. For example, the acid-fast bacilli (nos. 54, 55) were sandwiched between Gram-negative bacteria, and could be seen to be misplaced because of the pale band which they made through that part of the diagram which indicated comparisons between them and the Gram-negative bacteria: this indicated low similarity with the Gram-negative bacteria. Similarly no. 8, a streptococcus, was placed among the Gram-negative bacteria, and this was also a misplacement, as was shown by a dark band across the left-hand side of the diagram (indicating high similarity with other Gram-positive bacteria) and a pale band across the right-hand side (indicating low similarity with Gram-negative bacteria).

The table was therefore rearranged, and although this is to some extent an arbitrary step, it rests on the rational basis that mean S values between groups of strains are best for obtaining a satisfactory order of the groups; the sorting procedure employs the highest S values for determining the order only because this is mathematically a simpler procedure. It is perhaps not surprising that some rearrangement was needed with such a varied collection of strains. It is not possible to arrange such a collection in a perfect order, since relations between organisms are multidimensional, as has been pointed out in the description of this method. The organisms nos. 1–3 have been placed on the extreme top and left owing to their closer similarity to Gram-positive organisms than to the Gram-negative. The order given by the sorting procedure is given in the legend to Fig. 1. The rearranged table is shown diagrammatically in Fig. 1, and this forms the basis for the section on Results. The figure is shaded at 10 % levels of S , which is as close as is justified, since the error of an individual S value is at least ± 10 %.

Subgroups. Within group 1 are three subgroups, subgroup 1*a* consisting of several streptococci (nos. 4–6), subgroup 1*b* (nos. 9–11) containing two micrococci and surprisingly also a diphtheroid organism and subgroup 1*c* (nos. 13, 14) containing *Staphylococcus aureus* and *S. saprophyticus* (the latter species contains most of the organisms known to medical bacteriologists as *S. albus*). The other organisms of group 1 do not form clear-cut subgroups: these are *Corynebacterium pyogenes*, *Erysipelothrix rhusiopathiae*, *Listeria monocytogenes*, *Staphylococcus lactis*, *Aerococcus viridans* and a group O streptococcus. They may be subgroups of their own, or may be intermediate forms between other subgroups. There is some overlap between the staphylococci in subgroup 1*c* and the Gram-negative group 2.

Within group 2 is a large subgroup, 2*a*, containing many of the enterobacteria (nos. 15–29), but excluding *Shigella dysenteriae* and *Proteus*. It is divided somewhat indistinctly probably because of the small number of features employed, into sections such as *Escherichia*, *Salmonella* and *Vibrio*. Subgroup 2*a* also contains *Pasteurella pestis*. It overlaps with subgroup 2*b* (nos. 30–36) consisting of *Actinobacillus lignieresii*, *Pasteurella septica*, *Brucella melitensis* and *Neisseria gonorrhoeae* and perhaps also *Shigella dysenteriae* type 1 (Shiga's bacillus). The last-named shows little affinity to strains of subgroup 2*b* and may perhaps rank as a subgroup of its own; it has been shown thus in Fig. 2. Subgroup 2*c* contains *Pseudomonas aeruginosa* and one strain of *Loefflerella pseudomallei* (no. 39). The other strain (no. 29) of the latter organism is placed in subgroup 2*a*, which is a finding we cannot explain. Strain 29, isolated from a sheep, possesses the biochemical properties of a recently isolated strain of Whitmore's bacillus, but is agglutinated only to a low titre by an antiserum known to agglutinate three other strains of Whitmore's bacillus isolated from human cases of melioidosis. Strain 39 is agglutinated to titre by the serum, but has lost a number of biochemical properties, which is a common occurrence on subculturing this organism. Presumably one or the other should be regarded as aberrant, but we cannot say which.

Subgroup 2*d* (nos. 40–43) contains species of *Bacillus*. Subgroup 2*e* (nos. 44–46) contains mesophilic strains of *Chromobacterium*, and also *C. iodinum* Davis, although this may be a mistake, as the survey of Sneath (1957*b*) did not indicate such a close relationship. Subgroup 2*f* (nos. 47, 48) contains species of *Proteus*. Strains of group 2 which appear to be unlike all other strains of group 2 are nos. 49–51. They are a psychrophilic strain of *Chromobacterium*, the glanders bacillus and *Pseudomonas diminuta*. They presumably represent separate subgroups of group 2.

There are insufficient strains of groups 3–5 to reveal subgroups, but some relations to other groups may be mentioned. The two strains of *Corynebacterium diphtheriae* (group 3, nos. 52, 53) appear to be intermediate between groups 1 and 2, though not closely similar to either. The high value for the comparison between *Jensenia canicruria* (no. 56) and *Staphylococcus afermentans* (no. 11) appears to be fortuitous, since *Jensenia* is not very similar to any other strain of group 1. It has been placed in group 5, since it seems closer

to the acid-fast bacteria; it may perhaps be a form connecting the acid-fast and the Gram-positive bacteria. Two strains of *Clostridium* were also analysed, but they are not included since, being obligate anaerobes, they could not be validly compared with the other strains. However, they did not show a close similarity to any of the other strains or to one another. The two strains were *Clostridium butyricum* NCTC 7423 and *C. botulinum* NCTC 7272.

Summarizing the figure into a taxonomic tree

It is not possible to express all the relations indicated in Fig. 1 in the few dimensions of a taxonomic tree, and it is doubtful whether the data used here would justify any very elaborate taxonomic tree. Only a rough figure (Fig. 2) is given which shows the main outlines of the classification which is indicated by this study. This figure was drawn from the results of taking mean *S* values of the groups and subgroups in the same way as in the previous survey (Sneath, 1957*b*).

Comparison with other surveys

We can compare the results of two surveys if we have a number of strains which were used in both surveys, and it is then possible to calibrate roughly one *S* value scale with the other. A crude but simple method for doing this is shown in Table 3 and Fig. 3, which relate the present survey with that of

Table 3. Comparison of *S* values in two surveys

The *S* values for comparisons between strains used in both surveys are tabulated below. Table 3*a* shows the *S* values found in the present survey, and Table 3*b* shows those found in the survey of Sneath (1957*b*). The strain numbers are those used in the present paper.

(a) % *S* values found in the present survey

Strain number	Strain number							
	10	23	24	38	44	45	46	50
10	100							
23	50	100						
24	54	77	100					
38	44	58	47	100				
44	55	60	56	67	100			
45	58	62	62	55	82	100		
46	59	60	54	67	73	63	100	
50	50	61	61	58	65	63	59	100

(b) % *S* values found in survey of Sneath (1957*b*)

Strain number	Strain number							
	10	23	24	38	44	45	46	50
10	100							
23	32	100						
24	33	95	100					
38	38	50	54	100				
44	43	63	62	57	100			
45	38	57	54	47	82	100		
46	63	30	29	39	34	31	100	
50	33	44	41	56	49	42	29	100

Sneath (1957*b*). It is seen from Fig. 3 that the main distortion between the two *S* value scales lies in the region 30–70 %.

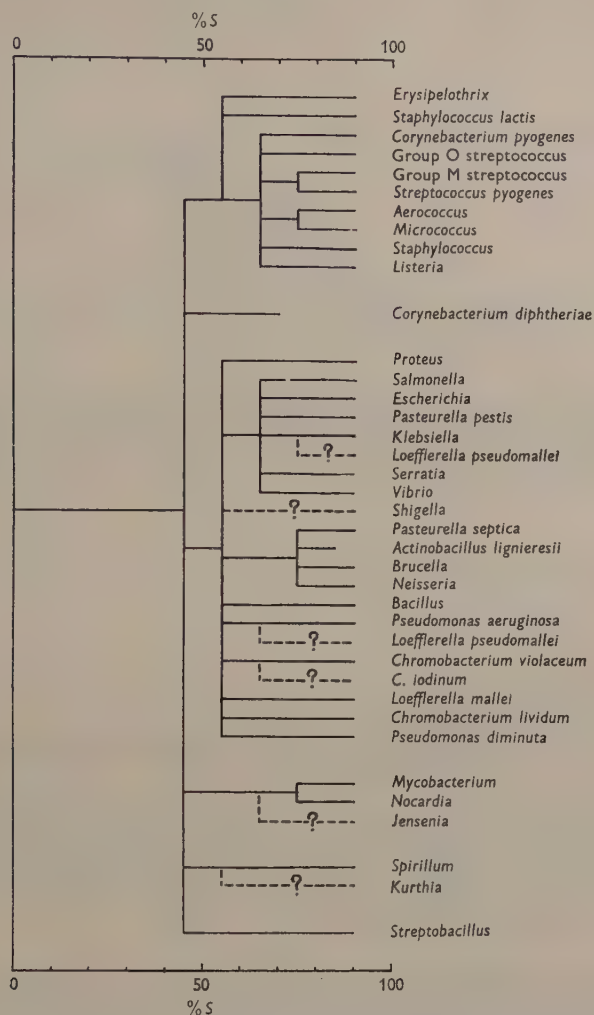


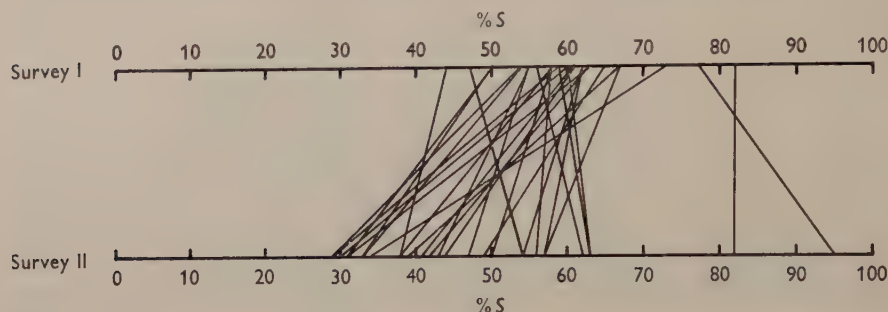
Fig. 2. Taxonomic tree showing the main outlines of the classification suggested by this study.

DISCUSSION

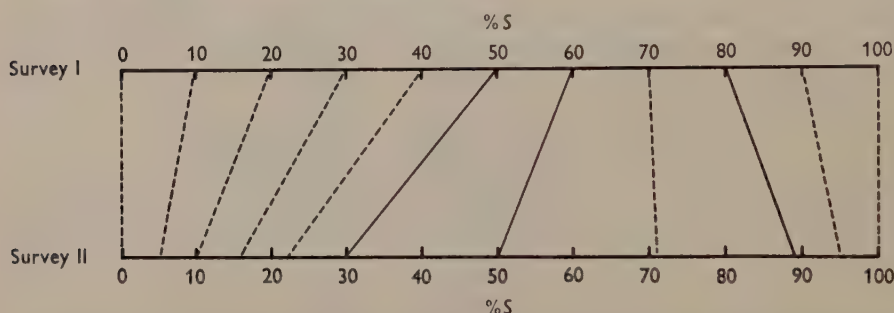
Limitations of the present survey

Old laboratory records are clearly not as reliable as data from a set of tests put up at one time and compared with one another by a single observer. In the records used here it was noted that there were generally some discrepancies in the features recorded at different examinations of a strain, due presumably to slight differences in media, technique and scoring of the results, but since the Collection consists of freeze-dried cultures there were few changes due to

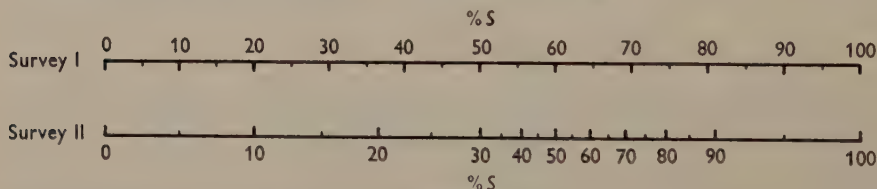
variation on subculture. The number of discrepancies suggests that there is an experimental error of at least 5 % in scoring the features used for the analysis. Therefore, the *S* values cannot be relied upon within 10 % either way. This error could be virtually eliminated by more careful taxonomic study.



- (a) Method of calibration. A line is drawn from the *S* value found in one survey for the comparison of a particular pair of strains to the *S* value found in the other survey for the comparison of the same pair of strains (see Table 3). All the comparisons which were made in both surveys are similarly entered.



- (b) The approximate mean correspondence between the two scales of *S* is obtained by drawing lines from one scale to the other based on the mean slopes of the lines in figure 3(a). The *S* values of 0 and 100 % are the same on both scales. Endashed lines are only presumptive, as they are not based on information in figure 3(a).



- (c) From figure 3(b) the two scales are calibrated against each other by distorting one of them.

Fig. 3. Calibration of the *S* value scale of the present survey (I) with that of the survey of Sneath (1957b) (II).

There is also the 'error' which is due to the fact that even in the most homogeneous groups of organisms there is some variation in features from strain to strain. Some strains will therefore show higher *S* values than others when they are compared with a strain from another homogeneous group. This is

probably the explanation for the high *S* value for the comparison between *Jensenia canicruria* and *Staphylococcus saprophyticus*.

Yet another cause of error arises because in a collection of diverse bacteria some groups are routinely examined by certain tests but not by others. Thus, the enterobacteria had usually been examined for amino acid decarboxylases but not for haemolysins, while streptococci had been tested for haemolysins but not for decarboxylases. This would cause some discrepancies between the results obtained here and those which would have been obtained if both groups had been tested in both sets of tests (as would be ideal), although there are reasons for believing that the discrepancies would not be large. The effect of this cause of error is to produce slightly different *S*-value scales for the different groups, and, as can be seen from Fig. 3, while the general conclusions on relations between taxa would be little affected, there might be significant discrepancies in the taxonomic rank and in the relations of individual strains.

For these reasons we do not propose any taxonomic revisions on the results described here, and regard the latter only as pointers for more detailed work. For such work a much greater number of features will be needed to obtain a sound basis for bacterial taxonomy.

The taxonomic arrangement suggested by this study

The results of this study may be compared with a conventional classification by comparing Fig. 2 with Table 2. Those who have studied the Gram-positive cocci will not be surprised to find such a high similarity between streptococci, micrococci and some diphtheroids, but it is unexpected that *Bacillus* is placed with the Gram-negative organisms. The closeness of *Pasteurella septica*, *Brucella*, *Neisseria* and *Actinobacillus lignieresii* to the typical enterobacteria, and the dissimilarity of *Proteus* are also unexpected.

The strain of *Spirillum rubrum* is believed to be the original strain of Esmarek. Whether it was capable of photosynthesis when first isolated is not known, but if so the similarity to *S. mancuniense* suggests that there may be a close relation between the spirilla and the Athiorhodaceae, in which *S. rubrum* is now commonly placed (under the name *Rhodospirillum rubrum* (Esmarek) Molisch).

The only representative of the genus *Shigella* which was studied was Shiga's bacillus which is probably the least typical of all shigellas although it is the type species. It is catalase-negative, grows more feebly and is less active biochemically than the other members (serotypes) of the genus. This may explain why *Shigella*, in this survey, is not as closely related to *Escherichia* as would be expected on other grounds. *Proteus* spp. have several features not often found in the Enterobacteriaceae but two of these, gelatinase production and swarming, are easily lost on subculture. Thus the old stock culture of a *Proteus* species more closely resembles other enterobacteria than do freshly-isolated strains.

Taxonomic rank

It is clear that there are discrepancies between the rank indicated by the *S* values and current usage. For example, *Salmonella*, *Escherichia* and *Vibrio*

seem to be of lower rank than *Streptococcus* and *Bacillus*. If the latter are retained as genera the former should logically be reduced to the rank of species; alternatively, *Streptococcus* and *Bacillus* could be subdivided into several genera. There can be no absolute criterion of taxonomic rank (as one man's meat is another's poison, so a genus to one will be a family to another), but if certain examples of rank are defined, others can be adjusted to be in harmony. Agreement will not be reached unless rank is assessed on a quantitative basis (whether by the methods used here or by others) and international agreement will be necessary for this.

Divisions into taxa

Tables of *S* values do not afford a good guide to whether the strains fall into clear-cut taxa without intermediate forms, since correlations between features (upon which clear-cut taxa are based) may be obscured by the effect of features which are variable from strain to strain. The correlations can be found by examining the original table of strains versus features. This is outside the scope of the present survey, but it may be noted that strains of group 1 generally are non-motile, Gram-positive, coccoid, penicillin-sensitive, seldom produce gas from glucose and have complicated requirements for growth factors, while strains of group 2 generally show the opposite characteristics.

The results suggest that it might be profitable to study the streptococci, micrococci and diphtheroid bacilli as a whole, rather than as separate genera, and also to include genera such as *Pasteurella* and *Vibrio* in surveys of the enterobacteria. In an intensive study of a particular group it would help to keep the taxonomy of bacteria in perspective (by providing criteria for higher ranks) if a few standard strains from several of the main groups were included. By limiting their objectives taxonomists have often failed to see the wood for the trees; for instance it is only recently that the similarity between *Corynebacterium pyogenes* and streptococci has become apparent (Cummins & Harris, 1956), a relation which this survey has confirmed.

It is well known in biology that a group of organisms can be sorted or arranged in many ways, and like anything else which is essentially a matter of arrangement, the arrangements cannot be true or false but only more convenient or less convenient for specified purposes. For 'general purposes' a 'general classification' is required, which Adansonian principles will provide. Such general classifications cannot be perfect for special purposes. Orthodox taxonomy is in its basic concepts a 'general classification for general purposes'. The example given here shows how progress can be made on these lines in bacterial taxonomy.

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On the Infrared Absorption Spectrum of *Bacillus megaterium*

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SUMMARY: The infrared absorption spectra of the aerobic spore-forming bacilli are variable. Evidence is presented to show that, for *Bacillus megaterium*, the nature of the spectrum is related to the morphology of the bacteria, both showing a cyclic variation with time. The time scale of the cycle is affected by the temperature at which the cultures are incubated, by their population density and by the medium on which they are grown; it may be affected by induced drug resistance. Some of the characteristics of the spectrum of vacuolated vegetative organisms are due to the presence of an extractable substance which may be a polymer of β -hydroxybutyric acid. Difference spectroscopy is used to demonstrate the presence of this substance, its disappearance as the bacteria autolyse and the appearance of β -hydroxybutyric acid in the autolysed material. This technique is applied also to establish the state of dipicolinic acid in the resting spores of *B. megaterium*. Any application of infrared spectroscopy to the study of aerobic spore-forming bacilli must take account of the morphological state of the bacteria.

It has been demonstrated that, for strains representative of many genera, reproducible infrared absorption spectra can be obtained from films of bacteria if the conditions of growth, the methods of harvesting and specimen preparation, and the instrument conditions are carefully standardized (Thomas & Greenstreet, 1954; Riddle *et al.* 1956). The spectra of the aerobic spore-forming bacilli are not nearly so reproducible (Blackwood & Epp, 1957; Haynes, Melvin, Locke & Glass, 1958) and in some instances have more absorption bands than those of other genera. In our previous investigation (Greenstreet & Norris, 1957) it was found that the spectrum of *Bacillus megaterium* underwent a regular change preceding the formation of spores. The changes in spectrum of one species of *Bacillus* are much greater than the reproducible differences observed between other strains, species or genera. This fact makes it difficult to fit the aerobic spore-forming bacilli into a system of classification based upon their infrared absorption spectra.

It is the object of this paper to study the changes in spectrum of *Bacillus megaterium* and to show that they are connected with the morphological appearance of the organisms and with the presence in the organisms of substances which have been isolated and characterized. The relevance of these changes in spectra to the differentiation of the aerobic spore-forming bacilli is discussed.

METHODS

Those workers principally interested in differentiating bacteria (Randall, Smith, Colm & Nungester, 1951; Stevenson & Bolduan, 1952; Thomas & Greenstreet, 1954; Levine, Stevenson, Bordner & Edwards, 1955; Riddle *et al.* 1956; Greenstreet & Norris, 1957; Kenner, Riddle, Rockwood & Bord-

ner, 1958) have insisted upon the use of standardized procedures; we have tried to do the same. For most of the experiments Hills's glucose lactate agar medium (GL agar) was used (Hills, Belton & Blatchley, 1949). Tryptic meat agar (TM agar), tryptose agar and liquid potato casein yeast medium (potato CCY; Powell & Strange, 1956) were also used.

A laboratory strain of *Bacillus megaterium* was used for most of the experiments. A limited investigation was made of laboratory strains of *B. anthracis*, *B. cereus*, *B. pumilus* and *B. subtilis*. Seed was stored at 3° as a resting spore suspension and watch was kept for contamination. For an experiment requiring 100 plates (9 cm. diameter Petri dishes), the procedure for preparing samples of bacteria for infrared examination was broadly as follows. The inoculum for the plates was prepared by adding 0.75 ml. spore suspension (2.3×10^{10} spores/ml.) to 100 ml. liquid medium (tryptic meat broth) contained in each of four Erlenmeyer flasks of capacity 500 ml. These were shaken at 37° for 70 min. so as to produce a suspension of germinated spores or early vegetative organisms with a uniform population. That is to say, all the organisms were similar in appearance. The shaking machine operated at 90 cyc./min. and had a stroke of 3 in. The suspensions were then centrifuged at 1900 g for 30 min. in a refrigerated centrifuge, and the deposit was resuspended in a volume of distilled water or physiological saline such that a predetermined density was obtained. Each plate of the selected solid medium was inoculated with 0.5 ml. of this suspension. A viable count (Miles & Misra, 1938) was made on the suspension used to inoculate the plates. The details of the procedure were varied to suit particular experiments, but the inoculum for the plates was always of germinated spores or early vegetative organisms with as uniform a population as possible. After incubation for various periods at the chosen temperature the bacteria were washed off the plates, centrifuged and resuspended in saline. Some of the suspensions were examined alive and some after autoclaving at 20 lb./sq.in. for 30 min.

The morphology of the bacteria was assessed by microscopical examination of films. These were made from the suspension used to inoculate the plate cultures and from the various suspensions harvested from them immediately before killing. They were stained to demonstrate and distinguish spores (Powell, 1950) and to demonstrate fat globules (Hartman, 1940). Occasionally wet films were examined under phase contrast illumination, since in this way refractile granules are readily visible (Hewitt, 1951).

A fixed volume of the bacterial suspension was spread evenly on a silver chloride window of prescribed size and heated at 60° until a dry film was obtained. This film was placed in the sample beam of the spectrophotometer and a similar but clean piece of silver chloride in the reference beam. Some suspensions were freeze dried. The spectra of these were obtained by preparing pressed disks containing about 1 % of the freeze-dried material in A.R. potassium chloride (Ford & Wilkinson, 1954).

The absorption spectra were recorded between 2 and 15 μ as % transmission *versus* wavelength by using a Grubb-Parsons S4 double-beam spectrophotometer equipped with a rock-salt prism.

RESULTS

We have examined the effect of varying the following factors upon the spectrum and morphology of *Bacillus megaterium*: (1) duration of incubation of the cultures; (2) temperature of incubation; (3) population density; (4) nature of the medium; (5) induced drug resistance.

Variation of spectrum with duration of incubation of the cultures

Plates of GL agar were each inoculated with 0.5 ml. of a suspension (2×10^8 viable early vegetative organisms/ml.) of *Bacillus megaterium* and incubated at 37°. At intervals suspensions were prepared by washing off the growth from different plates. Dried films of these suspensions gave spectra (Fig. 1) which show a regular change depending upon the duration of incubation. The change consists of absorption bands appearing in the spectrum at 5.78, 7.68, 7.97, 8.46, 8.85, 9.11, 9.47, 10.22 and 12.10 μ . These bands increase in intensity, reach a maximum at about 5 hr. and then decrease as the dura-

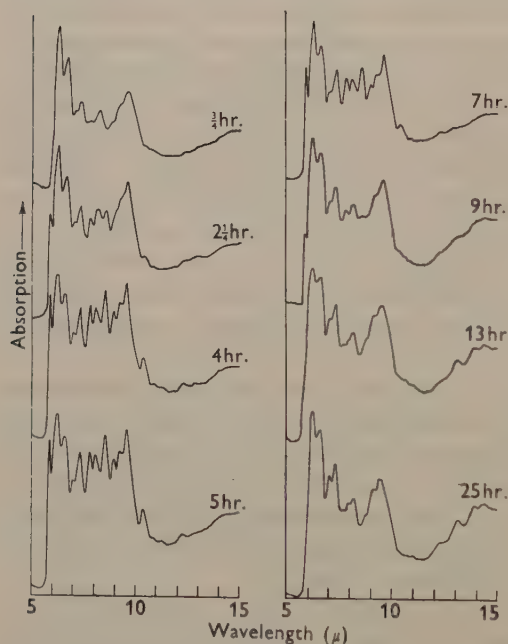


Fig. 1

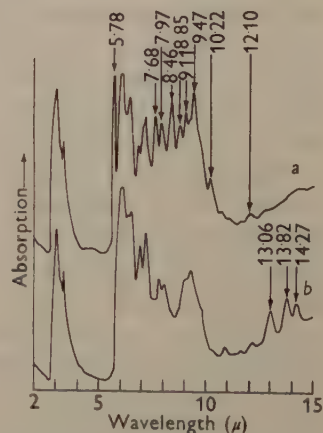


Fig. 2

Fig. 1. Variation of the infrared absorption spectrum of *Bacillus megaterium* with duration of incubation. Nine cm. diameter plates of Hills's glucose lactate medium were each inoculated with 10^8 early vegetative organisms and incubated at 37°. $\frac{1}{2}$ hr.=early vegetative organisms. $2\frac{1}{2}$ hr.=vegetative organisms showing signs of vacuolation. 4–5 hr.=highly vacuolated vegetative organisms. 7 hr.=prespores forming in cells. 9 hr.=prespores. 13–25 hr.=resting spores.

Fig. 2. Infrared absorption spectra of *Bacillus megaterium*. *a*, vacuolated vegetative organisms; *b*, resting spores. The absorption bands indicated are those referred to in the text: their wavelengths are given in microns.

tion of incubation increases. A spectrum with these additional absorption bands, which are indicated in Fig. 2*a*, will be described as complex. As these bands decrease in intensity new absorption bands appear at 13.06, 13.82 and 14.27 μ (Fig. 2*b*).

Films of the cultures stained with sudan black and with carbol fuchsin-methylene blue had the following appearances. After $\frac{3}{4}$ hr. incubation there was little detectable uptake of sudan black and with the spore stain the organisms appeared uniformly deep blue. After $2\frac{1}{4}$ hr. each organism contained several small granules which stained with sudan black; with the spore stain each organism contained several bodies which took up less blue stain than the remainder of the bacterium. At this stage the spectrum was becoming complex. Between 4 and 5 hr. the average length of the organisms decreased and they took up more sudan black than before although the granules were not so well defined. With the spore stain the organisms appeared to be highly vacuolated, the vacuoles occupying a large part of the bacterium. Organisms at this stage of morphological development will subsequently be referred to as vacuolated vegetative organisms. The spectrum of such organisms was complex (Fig. 1). After 7 hr. incubation the sudan black-staining granules had become very diffuse, the material staining blue with the spore stain was tending to concentrate at the poles and each organism contained one large pink-staining body. By 9 hr. there was hardly any uptake of sudan black, the prespore was well developed and stained a delicate pink with the spore stain and the remnants of the organism appeared as blue-staining material at the poles of the prespore. The spectrum of such organisms was less complex than at 5 hr. After 13 hr. the culture was entirely of free spores or of well-developed prespores which took up no sudan black. The spectrum was no longer complex and the bands shown in Fig. 2*b* were present. After 25 hr. incubation the culture consisted entirely of resting spores. This brief description agrees well with the more detailed observations of sporing cultures by Bayne-Jones & Petrilli (1933) and Lewis (1934).

Repeated experiments confirmed that there is an association between the shape of the spectrum and the morphology of the bacteria. Early vegetative organisms which stain uniformly with methylene blue do not have complex spectra. Vacuolated vegetative organisms have complex spectra. The prespore and resting spore do not have complex spectra, the spectrum of resting spores having the extra absorption bands at 13.06, 13.82 and 14.27 μ .

Cultures and spectra of *Bacillus anthracis* and *B. cereus* underwent a similar cyclic change. *B. subtilis* and *B. pumilus* also exhibited a cyclic change of spectrum with duration of incubation, but this differed from that for *B. megaterium*. At no stage was the spectrum complex, but the absorption bands at 13.06, 13.82 and 14.27 μ appeared in the spectrum of old cultures.

Changes in the spectra of *Bacillus megaterium* were characterized by the ratio of the strengths of two selected absorption bands. For this purpose strength is defined as the optical density at the band peak minus the optical density of the background at the same wavelength. The background is taken to be a straight line drawn tangential to the minima in the absorption spectra

near 5.5 and 11.5μ . The bands chosen were (1) a band at 6.10μ which appeared to remain constant throughout the cycle of events; (2) a band at 7.68μ which varied markedly with the duration of incubation and attained a maximum at the same time as the other eight bands in the complex spectrum.

The ratio α is defined by

$$\alpha = \frac{\text{strength of band at } 7.68\mu}{\text{strength of band at } 6.10\mu}.$$

Plotting the ratio α against duration of incubation for the spectra shown in Fig. 1 gives a curve with a maximum at 5 hr. Whenever cultures of *Bacillus megaterium* gave spectra with a high α the organisms appeared vacuolated with the spore stain and contained granules which stained with sudan black.

In an attempt to relate the change of α with the rate of growth of *Bacillus megaterium*, spores were inoculated into shaken potato CCY and at intervals the culture was sampled. The dry weight and α for each sample were plotted against time. In the early stages of the logarithmic phase of growth, when the bacteria stained uniformly with methylene blue, α was small; in the later stages, when the organisms were both larger and increasingly vacuolated, α increased. The dry weight and α each reached their maximum at about the same time. After this α fell rapidly whereas the dry weight fell slowly.

Variation of spectrum with temperature of incubation of culture

A large number of plates of GL agar were each inoculated with 0.5 ml. of a suspension (4×10^7 viable early vegetative organisms/ml.) of *Bacillus megaterium*. Half of these plates were incubated at 37° and half at 20° . Cultures incubated at the two temperatures were harvested at intervals until spores were formed. The values of α obtained from the spectra, when plotted against duration of incubation, gave curves of similar shape for each temperature, but with the maxima occurring after different periods of incubation: cultures grown at 37° had peak α at $5\frac{1}{2}$ hr., whereas cultures grown at 20° had peak α at 24 hr. (Fig. 3). Bacteria from the cultures which gave maximum α at the two temperatures were alike in appearance and could be described as vacuolated vegetative. The bacteria grown at the lower temperature remained longer at the stage of development at which they stained uniformly with methylene blue. Similar results were obtained when the experiment was repeated at 37° and 23° .

Variation of spectrum with population density

Plates (9 cm. diameter) of GL agar were inoculated evenly with 10^8 , 10^7 , 10^6 , 10^5 , 10^4 and 10^3 organisms/plate and incubated at 37° . After 10 hr. incubation confluent growth was obtained on the plates with inocula of 10^5 or greater. Curves of α versus duration of incubation for cultures grown from the different inocula are shown in Fig. 4. Similar curves were obtained when tryptose agar was used instead of GL agar. As the inoculum decreased so the time required for α to reach a maximum increased. Organisms on the plates with the smaller inocula remained in the early vegetative stages of growth

longer than those on plates with a larger inoculum. At peak α the bacteria had the same morphological appearance for all inocula. The effect that a change of inoculum can have upon the assessment of spectra is best demonstrated by an example. After $10\frac{1}{2}$ hr. incubation, cultures grown from inocula of 10^6 and 10^5 organisms/plate had almost identical spectra which had the same value of α . Three hours later spectra from the two cultures were very

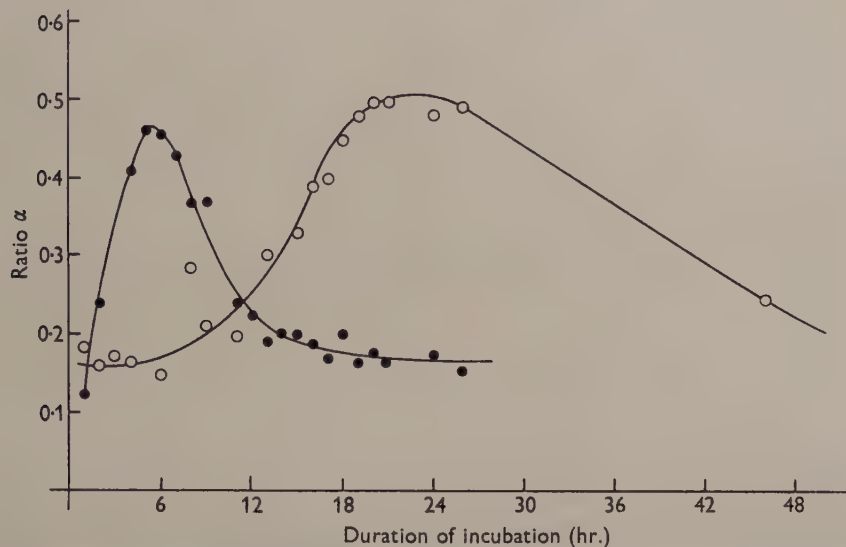


Fig. 3. Curves showing the change of α with duration of incubation for *Bacillus megaterium* grown on Hills's glucose lactate medium. ○—○ at 20°; ●—● at 37°; the inoculum was the same for all plates.

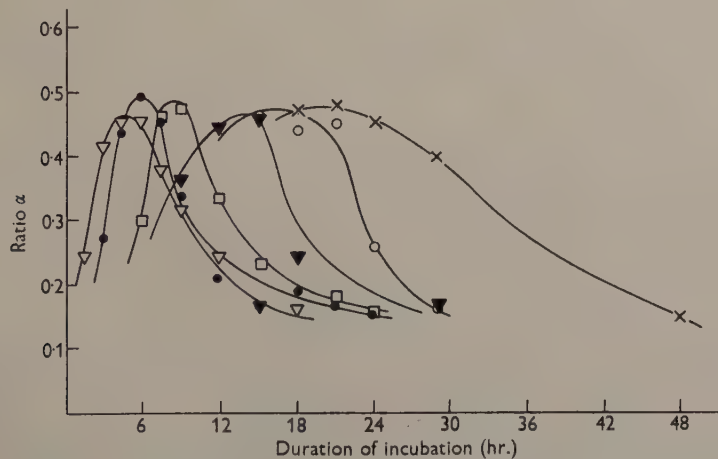


Fig. 4. Curves showing the change of α with duration of incubation for *Bacillus megaterium* grown on Hills's glucose lactate medium at 37°. The inoculum was varied: ▽—▽, 10^8 ; ●—●, 10^7 ; □—□, 10^6 ; ▼—▼, 10^5 ; ○—○, 10^4 ; ×—×, 10^3 viable organisms/plate (9 cm. diameter).

different indeed. The spectrum of the culture resulting from the inoculation of 10^5 organisms/plate had all the absorption bands shown in Fig. 2a and α was at its peak, whereas the spectrum of the culture from the 10^6 inoculum lacked these bands and gave a low value of α . A tenfold change in inoculum thus had a great effect upon the rate of development of the culture.

Variation of spectrum with medium

We used GL agar for the above experiments because we found that *Bacillus megaterium* grew uniformly on this medium and spore formation proceeded at a uniform rate throughout the culture, i.e. microscopic examination at any time showed that the majority of the bacteria in the culture had reached the same stage of development. Dilution of vacuolated vegetative organisms with non-vacuolated organisms reduced the value of α and made it more difficult to follow the changes described above.

Growth of *Bacillus megaterium* in shaken liquid potato CCY medium gave similar results to growth on plates of GL agar although in general the peak α for the spectra of bacteria grown in shaken liquid cultures was greater than that for cultures on solid media.

Bacillus megaterium grown on TM agar did not proceed beyond the vegetative phase of growth. The organisms when stained with the spore stain appeared vacuolated at first and then the vacuoles decreased in size but no spores were produced. The spectra showed a series of changes similar to that for growth on GL agar, the ratio α reaching a maximum when the bacteria were most vacuolated. When 1 % (w/v) glucose was added to the medium both the bacteria and the vacuoles appeared to be larger and the maximum value of α was reached after a longer period of incubation. The addition of 2 % (w/v) glucose to TM agar increased the effect produced by adding 1 %, but again no spores were formed.

On tryptose agar the morphology developed in a similar way to that on GL agar. Spores were produced after the vacuolated vegetative stage. The addition of glucose delayed spore formation. The greater the amount of added glucose the longer it took for α to reach a maximum.

Cultures from TM agar, tryptose agar and GL agar with added glucose harvested after a period of incubation greater than 10 hr. (inoculum 10^7 viable organisms/plate, temperature 37°) had a more complex spectrum than those harvested from the medium without added glucose.

Variation of spectrum with induced drug resistance

The tolerance of the parent strain of *Bacillus megaterium* to various antibiotics was increased by repeated subcultivation on gradient plates containing the antibiotics (Szybalski & Bryson, 1952). In this way tolerance to chloramphenicol, aureomycin, terramycin, streptomycin and bacitracin was increased. The relative tolerances of the parent strain and the derived strains to each antibiotic were assessed by a tube dilution method.

Cultures of the parent and of derived strains resistant to chloramphenicol, aureomycin, terramycin and streptomycin were harvested at intervals from

plates of GL agar, which had been inoculated with the same number of viable organisms/plate (5×10^6) and incubated at the same temperature. The spectra of these five series of samples were almost identical for any one period of incubation.

Small differences between spectra at any one time could be explained by differences in the morphology of the bacteria. The parent strain and the strain resistant to bacitracin were compared in a similar experiment. The curve of α plotted against duration of incubation for the bacitracin-resistant derivative was very different from that for the parent, its maximum appearing much later. When harvested after periods greater than 10 hr. the bacitracin-resistant strain always showed a more complex spectrum than the parent. When, however, the spectra were compared on the basis of morphology the peak value of α always coincided with the same stage of morphological development, i.e. vacuolated vegetative organisms. The difference between the spectra reflects a difference between the growth rates of the parent and derived strain. The difference in the rate of development of the cultures was obvious by even a cursory examination of the plates.

Chemical examination of vacuolated vegetative cells

The evidence presented by Burdon, Stokes & Kimbrough (1942) and Knaysi (1946) suggested that inclusions in vegetative organisms of some species of the genus *Bacillus* are fat-like. Attempts were made to extract such a substance from vacuolated vegetative organisms of *B. megaterium* by using acetone, benzene, carbon tetrachloride, chloroform, diethyl ether, ethanol and methanol. We did not succeed in extracting any fat or the component responsible for the extra absorption bands. In the course of making cell-wall preparations of vacuolated vegetative organisms by differential centrifugation of bacteria disintegrated by shaking with ballotini, a fraction was isolated which had an infrared absorption spectrum with peaks at all the wavelengths marked in Fig. 2*a*. The spectrum of the cell walls was almost entirely free from the bands due to this fraction, showing that it is contained within the bacterium and is not part of the wall.

Lemoigne (1923) found that suspensions of vegetative organisms in the *Bacillus subtilis* group became increasingly acid during autolysis owing to the formation of β -hydroxybutyric acid. A study of this phenomenon led him to isolate from *B. megaterium* and from *B. mesentericus* a substance which he referred to as 'lipide Y' and which he concluded was a polymer of β -hydroxybutyric acid (Lemoigne, 1926). He eventually succeeded in isolating two different products with melting points of 154° and 120° and he suggested that they were both polymers of β -hydroxybutyric acid but with different degrees of polymerization.

We have used one of his methods to isolate from vacuolated vegetative organisms of *Bacillus megaterium* a substance which corresponds with his 'lipide Y'. For convenience we shall refer to the material we isolated as the 'lipid'. The mean yield was 16 % of the dry weight of the bacteria when these were grown on GL agar and 19 % in potato CCY.

The physical and chemical properties of the lipid isolated from our strain

were similar to those described by Lemoigne for the 'lipide' he isolated; our lipid contained $<0.02\%$ phosphorus. The only exception was the melting point of the lipid. Our product melted at 165° , whereas Lemoigne's products melted at 154° and 120° . Lemoigne said that the 'lipide' is a polymer of β -hydroxybutyric acid and the infrared spectrum is not at variance with this suggestion. Perhaps the product isolated by us had a higher degree of polymerization than either of those isolated by Lemoigne.

Difference spectroscopy applied to Bacillus megaterium

Spectra of vacuolated organisms and germinated spores have much in common (Fig. 1). By placing a specimen of vacuolated organisms in the sample beam of a double-beam instrument and germinated spores in the reference beam it is possible to obtain the spectrum of the difference between the two

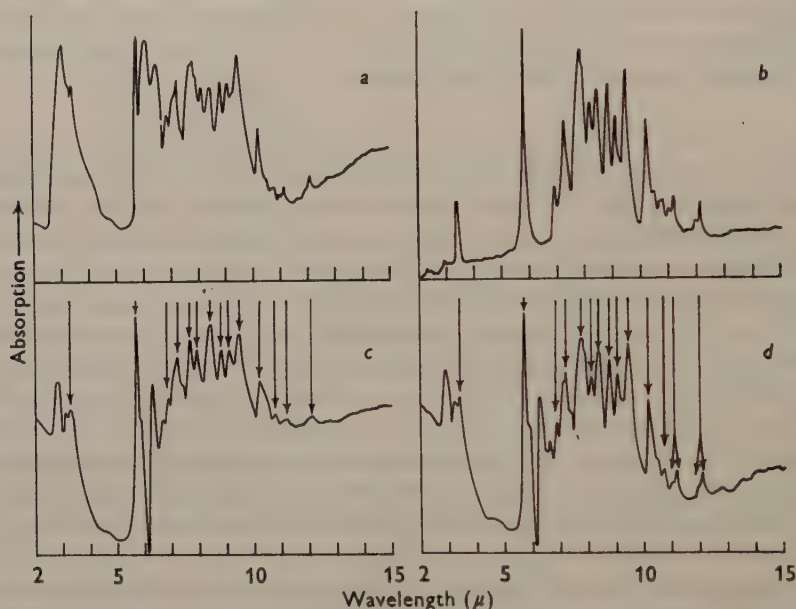


Fig. 5. Infrared absorption spectra of *Bacillus megaterium*. *a*, type B spectrum of vacuolated vegetative organisms; *b*, the lipid isolated from vacuolated vegetative organisms (dried film on silver chloride); *c*, the difference spectrum between vacuolated vegetative organisms which have a type A spectrum, and germinated spores; *d*, the difference spectrum between vacuolated vegetative organisms which have a type B spectrum, and germinated spores. The absorption bands marked with an arrow in *c* and *d* appear in the spectrum of the extracted lipid *b*.

specimens. This technique and its limitations have been discussed by Robinson (1952); Bellamy (1953); Martin (1957) and Bartlet (1957). The difference spectrum between vacuolated vegetative organisms and germinated spores is shown in Fig. 5*c*, the absorption bands marked with an arrow appear in the spectrum of the extracted lipid (Fig. 5*b*). A large part of the difference between vacuolated vegetative organisms and germinated spores can thus be attributed

to the presence of the lipid in the vacuolated organisms. We used germinated spores as our reference material because they are almost free from calcium dipicolinate and lipid. Absorption bands attributable to the lipid do not appear in the spectra of our strains of *Bacillus subtilis*. This is in accord with Lemoigne's report that he was able to extract only 0.029 % (dry weight) of his 'lipide' from this species.

We observed that the spectra obtained from a given sample of *Bacillus megaterium* when freshly prepared and after being kept for a time may not be identical. This effect occurs when dry films of bacteria on silver chloride are stored for more than 12 hr.; it occurs also in some heat-killed suspensions stored at 3°. The factors which determine whether or not a suspension changes on storage have not yet been found. Freshly harvested vacuolated organisms, whether alive or killed, have spectra which are like that in Fig. 6*a* and for convenience this will be called a type A spectrum. The spectrum of stored bacteria can change to that shown in Fig. 5*a*; this will be referred to as a type B spectrum. The difference spectrum between organisms giving a type B spectrum and germinated spores (Fig. 5*d*) is similar to that of the extracted lipid. The bands marked with an arrow in Fig. 5*d* appear in the spectrum of the extracted lipid.

Artificial mixtures of lipid and germinated spores have been made and from these it was shown that up to a concentration of 50 % the ratio α is proportional to the amount of lipid in the preparation. The ratio α can thus be used to estimate the proportion of lipid in vegetative organisms. It has already been seen that α achieves a greater maximum for bacteria grown in shaken liquid medium than for bacteria from plate cultures. Thus growth in shaken liquid medium results in the production of a greater proportion of lipid. This is in accordance with our yields of extracted lipid and with the work of Lemoigne (1946).

When a washed suspension of vacuolated organisms is allowed to autolyse the suspension becomes acid and loses all the bands characteristic of the lipid (Fig. 6*b*). The acidity, according to Lemoigne, is due to depolymerization of the 'lipide' to β -hydroxybutyric acid. This is confirmed by the difference spectrum between autolysed vacuolated vegetative organisms and germinated spores (Fig. 6*c*) which contains nearly all the absorption bands in the spectrum of β -hydroxybutyric acid (Fig. 6*d*).

Powell (1953) observed that when resting spores of *Bacillus megaterium* germinated, there appeared in the medium a large amount of the calcium salt of pyridine-2:6-dicarboxylic acid (dipicolinic acid). We have obtained the spectra of the free acid, its sodium salt, its calcium salt (Fig. 7*d*) and its monoethyl and diethyl esters. The latter were examined because of the suggestion by Perry & Foster (1956) that the dipicolinic acid is present in the spore as the monoethyl ester.

The spectra of resting and germinated spores of *Bacillus megaterium* are shown in Fig. 7*a* and *b*. The difference spectrum between resting and germinated spores (Fig. 7*c*) is much more like the spectra of the calcium and sodium salts of dipicolinic acid than that of the free acid or of either of the esters. Since

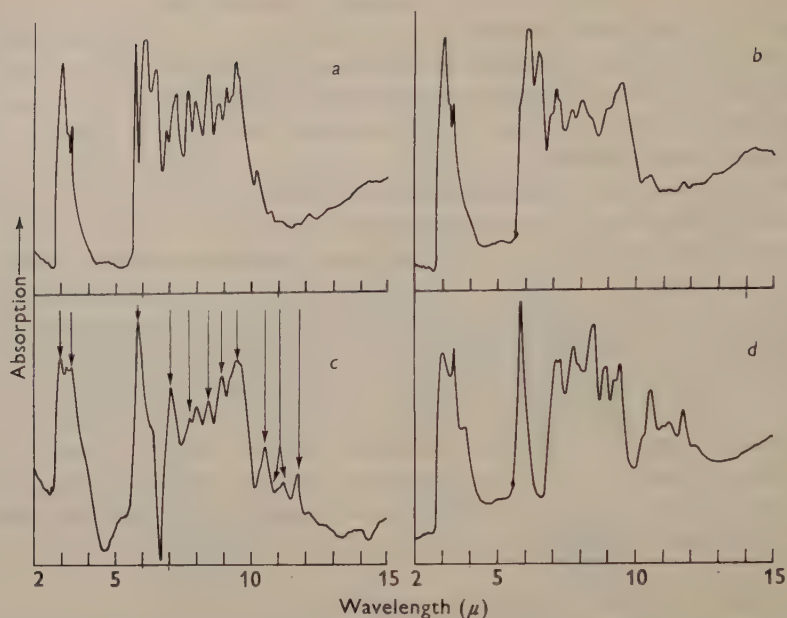


Fig. 6. Infrared absorption spectra: *a*, type A spectrum of vacuolated vegetative organisms (*Bacillus megaterium*); *b*, autolysed vacuolated vegetative organisms (*B. megaterium*); *c*, the difference spectrum between autolysed vacuolated vegetative organisms and germinated spores (*B. megaterium*); *d*, β -hydroxybutyric acid (film of pure liquid between sodium chloride plates). The absorption bands marked with an arrow in *c* appear in the spectrum of β -hydroxybutyric acid *d*.

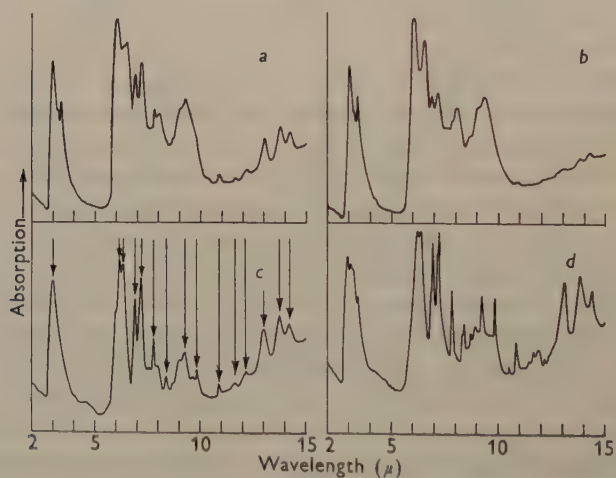


Fig. 7. Infrared absorption spectra: *a*, resting spores of *Bacillus megaterium*; *b*, germinated spores of *B. megaterium*; *c*, the difference spectrum between resting and germinated spores of *B. megaterium*; *d*, calcium salt of dipicolinic acid (potassium chloride pressed disk). The absorption bands marked with an arrow in *c* appear in the spectrum of the calcium salt of dipicolinic acid *d*.

calcium dipicolinate appears in the medium it is likely that dipicolinic acid is present in the spore largely as the calcium salt. If the resting spore contains the ester then it is in very small amounts.

DISCUSSION

The infrared spectra of *Bacillus megaterium* undergo a cyclic change and are most complex when the organisms of the sample show maximum vacuolation when stained with the spore stain and maximum uptake of sudan black. The main effect of varying parameters such as the medium, temperature of incubation, inoculum size and resistance to drugs is to alter the time scale of the development of the culture.

The curves of Fig. 4, which show the variation of the ratio α with duration of incubation for different population densities, the way in which the change of α with time is affected by the addition of glucose to the medium and the relationship between the change of α and the change of dry weight can be explained by one hypothesis, namely: that the bacteria do not accumulate lipid when they are actively dividing; that only when division has stopped do they increase in both size and lipid content. To test this explanation it will be necessary to observe the changes taking place in individual organisms, by using a culture chamber on a microscope (Powell, 1956).

At the stage of morphological development which we have described as vacuolated vegetative, the organisms contain several dark granules when stained with sudan black, and large refractile inclusions under phase-contrast illumination. The ratio α obtained from the spectra of such organisms shows that they contain a large amount of lipid. We have failed to extract the lipid from intact bacteria by fat solvents and have demonstrated that there is little or none in the cell walls. It seems likely that the vacuoles are the same as the granules which stain with sudan black, are the refractile bodies, and contain the lipid within the bacterium. The identity of the vacuoles and the granules has been suggested by Burdon, Stokes & Kimbrough (1942) but still awaits proof.

It appears that in the extraction process and in the ageing of the bacteria some change takes place in the lipid component. The difference between type A and type B spectra is very much smaller than the difference between the spectra of vacuolated and non-vacuolated organisms. The process underlying the change and its possible connexion with the degree of polymerization of the lipid must be the subject of further work. The methods we used to extract the lipid are rather severe but many authors, among them Lemoigne (1926) and Starkey (1946), recognized that acid hydrolysis or mechanical rupture of the cell wall was a necessary preliminary to the removal of fat from the bacterium.

Kull & Grimm (1956) recorded the spectra of bacterial strains trained to be resistant to certain antibiotics and compared them with the spectra of the sensitive parent strains. They found that there were qualitative differences between the spectra of resistant and sensitive strains of *Bacillus megaterium* and *Mycobacterium tuberculosis*, whereas the spectra of the resistant variants

of several other species showed no significant differences from those of the corresponding parent strains. In view of the large changes in spectra which result from altering the cultural conditions and since Kull & Grimm did not describe their experimental technique in sufficient detail, it is difficult to assess the significance of their conclusions. Our experience suggests that their results can be explained by a change in the time scale of the development of the culture.

Blackwood & Epp (1957) grew *Bacillus megaterium* on a nutrient medium containing glucose and obtained infrared spectra of the resulting cultures. Their spectra are similar to our type A spectra yet they conclude that β -hydroxybutyric acid accumulates in the bacteria. Our difference spectra make it clear however, that in freshly harvested bacteria it is not β -hydroxybutyric acid which is responsible for the complexity of the spectrum, but the lipid in substantially the same form as is obtained by extraction. When the suspension contains the free acid the spectrum has lost its complexity (Fig. 6*b*).

Efforts made to bring the aerobic spore-forming bacilli into a system of classification based upon their infrared absorption spectra have not as yet been successful, although Blackwood & Epp (1957) hold out the hope that it may be possible. The large variations in the spectra of vegetative organisms which result from altering the conditions under which the bacteria are grown are much greater than any due to species or strain differences. Originally we thought that cultures grown on media which inhibit spore formation, such as tryptic meat (TM) agar, might be used for identification but as has already been seen cultures harvested from TM agar undergo the full change in spectrum.

In any sample of vegetative organisms of the aerobic spore-forming bacilli harvested from solid or liquid medium there is considerable diversity of morphological forms. As these different forms have different infrared absorption spectra the spectrum of the whole sample depends upon the proportions of the morphological forms in the specimen. *Bacillus megaterium* grown on glucose lactate agar is relatively uniform in appearance at any stage of the growth cycle, but other species grown under similar conditions are relatively heterogeneous. This heterogeneity is not easily controlled and the simple procedure that we used to obtain the spectra of strains representative of other genera is not adequate to give specimens which appear morphologically homogeneous. The method used by Halvorson (1957) may be necessary to obtain synchronous growth. The variability introduced into the spectra because of these effects is of the same order of magnitude as the differences between the spectra of species in other genera. The only reproducible stage in the growth cycle is the resting spore. We have found that the spectrum of washed resting spores is not influenced by the medium on which the spores are produced.

Most spore suspensions are contaminated to a variable extent with vegetative organisms, lysed material and cell debris. A small amount of such contamination has a noticeable effect: the spectra of washed spores and of spores slightly contaminated with vegetative organisms are apparently different. The spectra of pure clean resting spore suspensions may make differentiation between species possible. So far the practical difficulty of preparing pure clean suspensions has prevented us from deciding whether or not this may be so.

In the past numerous attempts have been made to classify the *Bacillus* group on the basis of fat storage (Burdon *et al.* 1942; Lemoigne, Delaporte & Croson, 1944; Imšenecki, 1945; Burdon, 1946). The validity of the method has recently been assessed by Smith, Gordon & Clark (1952) who concluded that this criterion must be used with discretion and not as a definite basis on which to separate certain species from others. Their method was entirely qualitative, being based upon fuchsin-stained films. The infrared method can be made quantitative and therefore may eventually be of some use in differentiating between species. We are forced to the conclusion therefore that the infrared method cannot yet be used as a basis of differentiating between species of the aerobic spore-forming bacilli.

We thank Dr D. W. Henderson, Director, M.R.E. for his advice and criticism. We are indebted to Mrs Joan F. Powell for supplying us with samples of freeze-dried bacteria and of pyridine-2:6-dicarboxylic acid and its derivatives; and to the Distillers Company Ltd. for a gift of bacitracin. We are grateful also to Mr F. A. Dark for the differential centrifugation which gave us cell wall and lipid samples, to Mr H. E. Wade for phosphorus estimations, to Mr S. Bailey for the preparation of the monoethyl ester of dipicolinic acid and to Miss Jean M. Scott and Mr A. A. Dunthorn for their technical assistance. Finally, we wish to thank Mr E. O. Powell for his constructive criticism of the work and the manuscript.

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Torulopsis ingeniosa n.sp., from Grass Leaves

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SUMMARY: An undescribed species of *Torulopsis* has been isolated from pasture grass leaves. It does not ferment sugars and assimilates glucose, sucrose, maltose, lactose, galactose and potassium nitrate. It is distinguished from *Torulopsis aeria* by the oval shape of its cells, mucoid cultures on solid media, and the ability to liquefy gelatin rapidly. It is proposed to name it *T. ingeniosa*.

Isolations of yeasts from the leaf surfaces of pasture plants (*Lolium perenne*, *Trifolium repens* and others) collected in various parts of New Zealand included a species which resembled *Torulopsis aeria* (Saito) Lodder in many respects. Cultures on solid media were yellowish cream in colour, there was no production of ascospores, ballistospores or pseudomycelium, and budding was on a narrow base. Sugars were not fermented, no starch was synthesized on the medium of Mager & Aschner (1947), and glucose, sucrose, maltose, lactose, galactose and potassium nitrate were assimilated. However, as the cells were oval rather than 'almost round' (see Fig. 1), and as the texture of cultures on solid media was soft to mucoid or fluid and not 'dull glistening, almost smooth' (Lodder & Kreger-van Rij, 1952, p. 413), some doubt was felt as to the taxon's identity with *T. aeria*.

Comparison of a strain from grass with the type culture of *Torulopsis aeria* from the Centraalbureau voor Schimmelcultures showed that they were two separate species. As the undescribed species was able to use a number of complex substrates as sole carbon sources and was proteolytic, amylolytic and pectinolytic, it is proposed to name it *T. ingeniosa*.

Torulopsis ingeniosa n.sp.

Cellulae in culturis novis ovoidae $(2.5-3.5) \times (4.0-7.5) \mu$. Cultura nova in agar Sabouraudi albida, mollis saepe fluida. Pseudomycelium nullum. Fermentatio nulla. In medio minerali cum glucoso, saccharo, maltoso, lactoso, galactoso crescit. Nitras kalicus assimilatur. In cultura (post unum mensem) cellulae magniores sint. Cellulae circumdata capsula mucosa angusta.

Cells from young cultures oval $(2.5-3.5) \times (4.0-7.5) \mu$. Growth on Sabouraud (glucose peptone) agar cream coloured to yellowish, soft to mucoid, sometimes fluid. No pseudomycelium is formed. Giant cells may occur in old cultures. A narrow capsule is demonstrable.

Fermentation: absent.

Sugar assimilation: Glucose + Lactose + (sometimes weak)
 Sucrose + Galactose + (sometimes weak)
 Maltose +

Assimilation of potassium nitrate: positive.

Biochemical tests were carried out by the methods of Lodder & Kreger-van Rij (1952).

Although cell shape and consistency of cultures on solid media were sufficient to show that *Torulopsis ingeniosa* was a different species from *T. aerea*, it seems advisable to include some supplementary character to distinguish the two taxa. Both the type strain of *T. aerea* and that of *T. ingeniosa* gave very weak growth with ethyl alcohol as a carbon source, and both species could split soluble starch and grow in a defined medium free from growth factors.

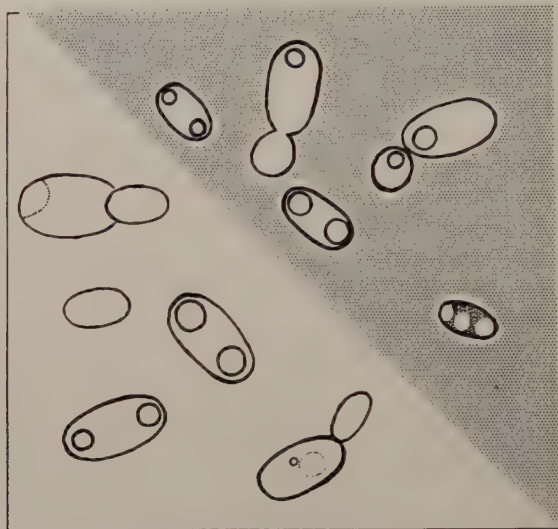


Fig. 1. Cells of *Torulopsis ingeniosa* showing narrow capsules. $\times 2500$.

When ferulic acid, vanillin and *p*-hydroxybenzaldehyde in 0.01 % concentration (w/v) were used as carbon sources, *T. ingeniosa* could assimilate all three, but *T. aerea* could use only ferulic acid. However, these aromatic compounds are not used routinely in the laboratory and growth in them is slow. All strains of *T. ingeniosa* tested caused stratiform liquefaction of gelatin within a week; the type strain of *T. aerea* had not liquefied gelatin after incubation for 3 weeks. It is therefore suggested that liquefaction of gelatin be used as a supplementary test for *T. ingeniosa*.

Theoretically there appears to be some danger of confusing *Torulopsis ingeniosa* with those strains of *Candida muscorum* di Menna which produce limited amounts of pseudomycelium. Biochemically the two species are very similar (di Menna, 1958 and to be published), and both produce creamy mucoid to fluid cultures on solid media. In practice, production of pseudomycelium in *C. muscorum* and lack of any such growth form in *T. ingeniosa* have always been sufficiently marked to prevent confusion. A more important distinguishing feature between the two species seems to be habitat. *T. ingeniosa* has been isolated from leaves of pasture grasses and, less frequently and in smaller proportions, from the soil beneath the grasses. *C. muscorum* has been found in rotted sphagnum in peat bogs and in forest soils.

The type culture of *Torulopsis ingeniosa*, TG 27, isolated in July 1957 from

grass leaves near Hamilton, New Zealand, has been deposited at the Centraal-bureau voor Schimmelcultures, Yeast Division, Delft, Netherlands, and at the Brewing Industry Research Foundation, Nutfield, Surrey, England.

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Dihydrostreptomycin and Anaerobiosis—Indirect Evidence for Two Sites of Action of Dihydrostreptomycin

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SUMMARY: The sensitivity of 4 strains of *Escherichia coli* and of *Aerobacter aerogenes* when growing under aerobic and under anaerobic conditions in heavily buffered enriched medium was compared. Each comparison covered a series of pH values from 5.5 to 8.0. A distinct pattern emerged for each group of organisms and this would appear to be related to the enzymic make-up of the organism with respect to carbohydrate metabolism and not to the pH developed in the medium during growth or to the growth rate of the organism. Comparison of the behaviour of *A. aerogenes* under identical conditions, but in a simple medium, suggests that dihydrostreptomycin is more effective against synthesizing than against energy-producing mechanisms.

The activity of streptomycin and dihydrostreptomycin is decreased under anaerobic conditions (Bondi, Dietz & Spaulding, 1946; Geiger, Green & Waksman, 1946; Hurowitz, Rosano, Blattberg & Rosenfeld, 1955; Williamson & White, 1956). Three suggestions have been advanced to explain this phenomenon. First, an altered environment, the direct effect of the incomplete catabolism of metabolites. This gives rise to acidic end-products, lowering the pH of the medium and thereby decreasing the effective concentration of the antibiotic (Geiger *et al.* 1946). Secondly, a slower rate of growth, for it has been stated that the faster an organism grows the greater its sensitivity; indeed there would appear to be a direct relationship (Hurowitz *et al.* 1955). Finally, 'the antibacterial action of streptomycin may be due to its ability to block some enzyme system, oxidative in nature, which is essential only to the growth of susceptible aerobic bacteria, an enzyme system which anaerobes do not possess' (Bondi *et al.* 1946). This idea could conceivably apply to facultative anaerobes if their functional enzymic pattern varies with the oxygen content of their environment. Of the many metabolic pathways that may be affected by streptomycin those concerned with carbohydrate breakdown have been most fully investigated. Interference with the aerobic metabolism of pyruvate by *Escherichia coli* was reported by Oginsky, Smith & Umbreit (1949) and that of anaerobic dissimilation by Barkulis (1953). The several pathways for the breakdown of pyruvate by *E. coli* and *Aerobacter aerogenes* are the same with the exception of that leading to the formation of acetylmethylcarbinol by *A. aerogenes*. Furthermore, this mechanism functions under both aerobic and anaerobic conditions. It was thought that by working under carefully controlled environmental conditions with organisms whose growth rate and general metabolic behaviour was known, some reason for the greater resistance to dihydrostreptomycin of a facultative anaerobe when growing anaerobically might be forthcoming.

METHODS

Organisms. Four strains each of *Escherichia coli* and of *Aerobacter aerogenes* were used, with the exception of *E. coli* NCTC 5928 and *A. aerogenes* NCTC 418, isolated from routine specimens of faeces.

Media. Two fluid media were used throughout.

(a) Enriched medium: glucose peptone m/6-phosphate which was made up from four component solutions: (i) Difco proteose peptone, 3.0 g.; KH_2PO_4 (Analar), 22.65 g.; distilled water to 950 ml. (ii) Difco proteose peptone, 3.0 g.; Na_2HPO_4 (Analar), 23.65 g.; distilled water to 950 ml. (iii) glucose (Analar), 24 g.; distilled water to 500 ml. (iv) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.0 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 2.5 g.; distilled water to 100 ml.

(b) Chemically defined medium: salt glucose m/6-phosphate. This was likewise made up from four component solutions: (i) NH_4Cl (Analar), 4.0 g.; KH_2PO_4 (Analar), 22.65 g.; distilled water to 950 ml. (ii) NH_4Cl (Analar), 4.0 g.; Na_2HPO_4 (Analar), 23.65 g., distilled water to 950 ml., and solutions (iii) and (iv) as above.

From each pair of phosphate solutions a series of mixtures covering the pH range 5.5–8.0 were prepared and these, together with the sulphate solution, were sterilized by autoclaving at 5 lb./sq.in. for $\frac{1}{2}$ hr. The glucose solution was sterilized by filtration.

Both final media consisted of 19 ml. phosphate mixture; 1.0 ml. glucose solution and 0.1 ml. sulphate solution (iv). After sterilization the pH value of each series of complete medium was determined on a Cambridge pH meter.

Dihydrostreptomycin. Crystalline dihydrostreptomycin sulphate (Glaxo) containing 1.0 g. equivalent of the base was dissolved in 10 ml. sterile distilled water to give a solution containing 100,000 μg . equivalents dihydrostreptomycin/ml. This was then used to prepare serial dilutions of the antibiotic. These dilutions, ranging from 1024 to 0.25 μg . equivalents dihydrostreptomycin/ml., were made up in the series of phosphate mixtures; a clean dry-sterilized pipette was used for each manipulation, so that any error due to 'carry-over' of fluid adhering to the outside of the pipette was eliminated. Such stock solutions, if not used immediately, were stored at 4°. Under such conditions of storage their potency, as assayed against *Klebsiella pneumoniae*, strain 41, remained constant for at least 1 month. No stock solutions kept for longer than 3 weeks were deemed suitable for accurate work.

Anaerobiosis. The anaerobic experiments were carried out in a MacIntosh and Fildes' anaerobic jar refilled, after evacuation, with hydrogen. To ensure a satisfactory anaerobic atmosphere the jar was evacuated and refilled with hydrogen three times. Reduced alkaline methylene blue was used as indicator of anaerobic conditions. When the number of tubes in an experiment necessitated the use of more than one jar, the jars were connected in series for evacuation and refilling.

Cleaning of glass-ware. All glass-ware was carefully cleaned by boiling in 10 % nitric acid followed by washing with glass-distilled water.

Determination of sensitivity to dihydrostreptomycin

Before each experiment the organism to be tested was first examined for purity and characteristic biochemical behaviour. It was then grown, by serial subculture on 3 successive days, in the medium and under the atmospheric conditions in which it was to be tested. A separate culture was prepared for each pH at which observations were to be made so that the enzymic constitution of the inoculum would be established and fixed. The last of the serial cultures was suitably diluted to give an inoculum yielding consistent growth in the controls (usually in the case of the glucose peptone medium 1/1000 and of the salt glucose medium 1/100).

Before determining the sensitivity to dihydrostreptomycin of the organism under examination growing aerobically and anaerobically a pilot aerobic experiment was set up to obtain an approximate estimate of its sensitivity throughout the pH range under consideration.

A range of doubling dilutions of dihydrostreptomycin straddling the sensitivity level obtained in the pilot experiment was then used for each pH value at which the organism was to be tested. 0.5 ml. samples of each dilution were then inoculated with 0.05 ml. of diluted culture. Groups of triplicate tubes were incubated aerobically and anaerobically in parallel. The results were read after 16 hr. incubation and then daily for 5 days. This was to enable any highly resistant cells in the inoculum to make themselves evident. In no case was the reading at 5 days greater than that after 16 hr. incubation. In other words the inoculum was uniform with respect to the degree of resistance to dihydrostreptomycin. For each pH a growth control, without dihydrostreptomycin, was included. In every experiment the density of growth was always greater under aerobic than under anaerobic conditions. Furthermore, oxygen did not appear to be a limiting factor in the aerobic experiments as aeration following the 16 hr. reading did not affect the result. After incubation each tube was subcultured on to nutrient agar to verify that turbidity was due to growth and to ensure purity of the culture.

Determination of rate of growth

Growth rate studies were made on *Escherichia coli* NCTC 5928 growing in peptone-glucose and on *Aerobacter aerogenes*, faecal strain R, growing in both media. Each organism was examined at pH 5.5, 6.5 and 7.5 when growing aerobically and anaerobically. The growth at pH 5.5 was too irregular to give consistent results as between replicates.

The aerobic studies were carried out in 50 ml. Erlenmeyer flasks containing a depth of medium equivalent to that in the aerobic culture tubes of the sensitivity experiments. The culture was transferred for turbidometric reading to a sterile $6 \times \frac{5}{8}$ in. test tube. Despite repeated disturbance in this manner a smooth growth curve was obtained suggesting adequate aeration. This belief was substantiated not only by a greater final density of growth than that reached in the parallel anaerobic cultures, but also by the failure of more vigorous aeration to stimulate further growth.

The anaerobic studies were made in specially adapted $6 \times \frac{5}{8}$ in. Pyrex test tubes which were evacuated, filled with hydrogen and clipped off. A nutrient broth contained in a similar tube and inoculated with the anaerobe *Clostridium welchii* was evacuated, filled with hydrogen and incubated in parallel as an indicator of anaerobic conditions. Direct turbidometric readings could thus be made on a culture growing anaerobically.

Each study, in parallel under aerobic and anaerobic conditions, was done in triplicate on three separate occasions.

RESULTS

The sensitivity to dihydrostreptomycin of the test organisms growing in glucose-peptone M/6-phosphate

The sensitivity to dihydrostreptomycin of the 4 strains of *Escherichia coli* and *Aerobacter aerogenes* growing under aerobic and anaerobic conditions is recorded in Table 1. One strain of each organism, *E. coli* NCTC 5928 and *A. aerogenes* R, was examined in greater detail.

From this survey it is seen that over a wide pH range the bactericidal activity of dihydrostreptomycin towards *Escherichia coli* is twice as great under aerobic as under anaerobic conditions. With *Aerobacter aerogenes* this greater activity is displayed only above pH 7.0.

The sensitivity to dihydrostreptomycin of Aerobacter aerogenes R growing in salt-glucose M/6-phosphate

The constituents of the medium in which the sensitivity of an organism to dihydrostreptomycin is determined may influence the result (Williamson, 1957). An examination of the same organisms growing in a simpler medium under otherwise identical conditions was next attempted. Comparison with the richer medium, however, was possible only with *Aerobacter aerogenes* R for this was the only organism found out of many examined which gave consistent growth under strictly anaerobic conditions at each pH used. In Table 2 the results obtained are compared with those using the glucose-peptone medium. The general pattern is the same in both media, although it will be noted that the test organism is at least four times as sensitive to dihydrostreptomycin when growing in the simpler medium. This result is in keeping with previous reports on the inhibitory effect of peptones (Lenert & Hobby, 1947; Foster & Pittillo, 1953). More important, the sensitivity of the organism is the same under aerobic and under anaerobic conditions.

The rate of growth of Escherichia coli NCTC 5928 and of Aerobacter aerogenes R

Hurowitz *et al.* (1955) reported that the sensitivity of *Escherichia coli* B to dihydrostreptomycin multiplied by the reciprocal of rate of growth was a constant (k). If this is so then the results obtained in glucose-peptone might be explained on this basis. However, a study of Table 3 shows that the value of k is not the same within those pairs showing a difference in sensitivity. As

might be expected the mean generation time of *Aerobacter aerogenes* R in the salt-glucose medium was slower (72 min.) than in the enriched medium and thus unrelated to the greater sensitivity to dihydrostreptomycin of this organism when growing in the simpler medium.

Table 1. *Sensitivity to dihydrostreptomycin of a number of strains of Aerobacter aerogenes and of Escherichia coli grown under aerobic and anaerobic conditions over a wide pH range in glucose peptone M/6-phosphate*

		Concentration of dihydrostreptomycin $\mu\text{g./ml.}$ inhibiting growth of							
		<i>A. aerogenes</i>				<i>E. coli</i>			
pH	Conditions of growth	R	NCTC 418	718	852	NCTC 5928	116	98	82
5.45	Aerobic	256	128	256	128	512	256	512	512
	Anaerobic	128	128	128	128	256	128	512	512
5.76	Aerobic	128	—	—	—	256	—	—	—
	Anaerobic	128	—	—	—	256	—	—	—
5.95	Aerobic	64	64	64	64	128	128	256	256
	Anaerobic	64	64	64	64	256	128	256	256
6.30	Aerobic	64	—	—	—	64	—	—	—
	Anaerobic	64	—	—	—	128	—	—	—
6.44	Aerobic	64	64	64	64	64	64	128	128
	Anaerobic	64	64	64	64	128	128	256	256
6.68	Aerobic	64	—	—	—	32	—	—	—
	Anaerobic	64	—	—	—	64	—	—	—
6.95	Aerobic	64	64	64	64	32	32	64	64
	Anaerobic	64	64	64	64	64	64	128	128
7.26	Aerobic	32	—	—	—	16	—	—	—
	Anaerobic	64	—	—	—	32	—	—	—
7.52	Aerobic	16	16	16	32	16	16	32	32
	Anaerobic	32	32	32	64	32	32	64	64
7.86	Aerobic	8	—	—	—	8	—	—	—
	Anaerobic	16	—	—	—	16	—	—	—
8.08	Aerobic	4	8	8	16	4	8	16	16
	Anaerobic	8	16	16	32	8	16	32	32

Each figure is the average of the results of at least three experiments carried out in triplicate.

Table 2. *Concentration of dihydrostreptomycin ($\mu\text{g./ml.}$) inhibiting the growth of Aerobacter aerogenes R under aerobic and under anaerobic conditions over a wide pH range*

		pH									
Medium	Conditions of growth	5.45	5.76	5.95	6.30	6.44	6.68	6.95	7.26	7.52	7.86
Glucose peptone	Aerobic	256	128	64	64	64	64	64	32	16	8
	Anaerobic	128	128	64	64	64	64	64	64	32	16
M/6-phosphate	Aerobic	128	64	32	32	16	16	8	4	2	1
	Anaerobic	16	16	16	16	16	16	8	4	2	1

Each figure is the average of the results of at least three experiments carried out in triplicate.

Table 3. Relationship of mean generation time (m) to sensitivity to dihydrostreptomycin (s) of *Aerobacter aerogenes* R and of *Escherichia coli* NCTC 5928 growing under aerobic and under anaerobic conditions in glucose-peptone M/6-phosphate

Organism	Conditions of growth	pH 6.5			pH 7.5		
		M.G.T. (min.)= m	Sensitive to dihydro- streptomycin ($\mu\text{g./ml.}$)= s	$k = s \times 1/m$	M.G.T. (min.)= m	Sensitive to dihydro- streptomycin ($\mu\text{g./ml.}$)= s	$k = s \times 1/m$
<i>A. aerogenes</i> R	{ Aerobic	40	64	1.60	35	16	0.46
	{ Anaerobic	44	64	1.45	37	32	0.86
<i>E. coli</i> NCTC 5928	{ Aerobic	52	64	1.23	44	16	0.36
	{ Anaerobic	56	128	2.29	46	32	0.70

Each figure is the average of the results of at least three experiments carried out in triplicate.

DISCUSSION

The acidic end-products of anaerobic growth cannot be responsible for the decreased activity of dihydrostreptomycin under the conditions here reported, since the buffering power of the phosphate was sufficient to prevent any alteration in the pH of the medium as a result of bacterial growth. Neither can it be due to a slower rate of growth of the organism under anaerobic conditions for this was almost the same in either air or hydrogen for both *Escherichia coli* NCTC 5928 and *Aerobacter aerogenes* R. These facts leave the assumption of a differentiation of the various metabolic systems of the bacterial cell by dihydrostreptomycin as the only possible explanation.

Before enlarging upon this hypothesis it is necessary to consider whether in the aerobic experiments oxygen could have become a limiting factor. This seems unlikely as aeration following the 16 hr. reading did not affect the absolute degree of resistance of the test organisms to dihydrostreptomycin. Furthermore, conditions approaching anaerobiosis can only occur following growth. In the sensitivity experiments the bactericidal value of dihydrostreptomycin was measured. Since low concentrations of dihydrostreptomycin were effective, when allowance is made for the adverse effect of the high hydrogen ion (Abraham & Duthie, 1946) and phosphate ion (Berkman, Henry & Housewright, 1947) concentration of the media, it seems reasonable to assume that its killing power was exerted upon growing and not upon resting organisms (Garrod, 1948) within the inoculum and therefore before aerobic conditions could be rendered even slightly anaerobic as the result of such growth.

It would appear reasonable to suggest that the action of dihydrostreptomycin upon *Aerobacter aerogenes* R when growing in the two media is entirely different. In the simpler medium which demands greater synthesizing activity from the organism, dihydrostreptomycin is both more active than in the richer medium and unaffected by the atmosphere in which growth takes place. This would suggest that the synthesizing activities of an organism are the most sensitive to the action of the drug. Since such activities are unrelated

to the atmosphere in which growth takes place no difference in sensitivity between the two atmospheres is shown. In the richer medium the need for synthesis is less and the site of action of dihydrostreptomycin might be assumed to be on less sensitive systems such as those yielding the energy necessary for synthesis and assimilation, i.e. those concerned with carbohydrate metabolism. At least three such systems are known to exist amongst the coliaerogenes group of organisms: one dependent upon air (the tricarboxylic acid cycle); one sensitive to or depressed by air (Utter & Werkman, 1944); the phosphoroclastic split; one independent of air (Silverman & Werkman, 1941; Stahly & Werkman, 1942); the acetylmethylcarbinol system. The last is present in *A. aerogenes* only and the upper limit of its activity is pH 7.2. This is the very pH below which any difference in sensitivity to dihydrostreptomycin dependent upon atmospheric conditions disappears. That such disappearance may be related to the functioning of this enzyme system and not to any other is suggested by the continuance of the difference in the case of *Escherichia coli*, an organism whose carbohydrate metabolism differs only in the absence of this system. If this explanation is acceptable then the greater resistance to dihydrostreptomycin exhibited by *E. coli* above pH 6.0 and *A. aerogenes* above pH 7.2 when they are growing anaerobically may well be due to the greater resistance to dihydrostreptomycin of the phosphoroclastic system than that of the aerobic tricarboxylic acid cycle.

The action of dihydrostreptomycin can thus be visualized as being on at least two levels, that interfering with synthesis and that affecting the energy-producing mechanisms. The latter is generally more resistant although the degree of resistance varies with the particular system functioning at the time.

The sensitivity pattern exhibited by *Aerobacter aerogenes* between pH 6.0 and 7.0 suggests two things. First, that the intracellular pH may well be constant since, despite increasing acidity of the external environment, the degree of sensitivity to dihydrostreptomycin does not vary. In the parallel experiments with *Escherichia coli* the level of resistance continues to rise with increasing acidity. This observation supports the view of Gale (1951) that the acetylmethylcarbinol system is a neutralization mechanism. Secondly, the acetylmethylcarbinol system must be either more resistant to dihydrostreptomycin than the other two systems or as resistant as the phosphoroclastic system otherwise a difference between the sensitivity of *A. aerogenes* growing under aerobic and under anaerobic conditions would still exist.

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Dependency on Medium and Temperature of Cell Size and Chemical Composition during Balanced Growth of *Salmonella typhimurium*

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SUMMARY: Cell mass, the average number of nuclei/cell and the content of RNA and DNA were studied in *Salmonella typhimurium* during balanced (steady state) growth in different media. These quantities could be described as exponential functions of the growth rates afforded by the various media at a given temperature. The size and chemical composition characteristic of a given medium were not influenced by the temperature of cultivation. Thus, under conditions of balanced growth, this organism exists in one of a large number of possible stable physiological states.

The variations in mass/cell are due to changes in the number of nuclei/cell as well as in mass/nucleus. An increase in the number of ribonucleoprotein particles at higher growth rates could, it appears, largely account for the increase in mass/nucleus. Calculations indicate that the rate of protein synthesis per unit RNA is nearly the same at all growth rates.

It is a classic observation that bacterial cells increase in size during the lag which precedes cell division in a newly-inoculated culture, and become smaller again during the period of declining growth (Henrici, 1928). It is also well known that increase in size and enrichment in ribonucleic acid go hand in hand (Malmgren & Hedén, 1947; Morse & Carter, 1949; Wade, 1952; Gale & Folkes, 1953). Previously, interest has been focused mainly on the striking difference between the small, non-dividing cells of an outgrown culture and the larger forms typical of rapid growth. Hence, cells are often described as 'resting' or 'exponentially growing' and these conditions implicitly considered to be alternative physiological states.

We have studied cells of *Salmonella typhimurium* during unrestricted, balanced growth in a variety of media and at different temperatures. The term 'cell' is used throughout this and the following paper to denote either a colony-forming unit or a microscopically visible rod. In both cases the unit may contain more than one nucleus. The terms 'unrestricted' and 'balanced' are defined in the discussion. In each case the growth rate, cell size, and the amounts of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) and the average number of nuclei/cell were determined.

These experiments show that a large number of physiological states exists, each of which is characterized by a particular size and chemical composition of the cells. *At a given temperature*, average mass, RNA, DNA and number of

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nuclei/cell can be described as exponential functions of the growth rate. In a given medium, cell size and composition are almost independent of the growth temperature. The characteristics of the cells would therefore seem to be determined primarily by the pattern of biochemical activities imposed by the medium.

The figures obtained for mass, RNA and DNA/cell permit estimates to be made of the quantities of protein and nucleic acids synthesized/cell/minute in different media at a given temperature. These calculations suggest that, over a wide range of growth rates, the amount of protein synthesized/minute is roughly proportional to the RNA content of the cell; or that, /unit of RNA, the number of protein molecules synthesized/minute is almost independent of the growth rate.

METHODS

Bacteria. The wild-type strain of *Salmonella typhimurium* used in this work was previously employed in this laboratory (Lark & Maaløe, 1954). The tryptophan-requiring mutant *try A-8* of *S. typhimurium*, kindly supplied by Dr M. Demerec, was used in the continuous-culture experiments.

Culture media. The media employed and the growth rates they supported are listed in Table 1. Amino acids and sugars were added after separate sterilization. All media were adjusted to pH 7.0.

Growth conditions. The organisms were grown in several hundred ml. volumes of medium through which air was constantly bubbled. Balanced growth was maintained by diluting with equal volumes of fresh medium at intervals corresponding to the average generation time. Before sampling, cultures were grown for several hours with frequent checks of the optical density in order to ensure that a constant growth rate had been established. As a rule, the optical density (at 450 m μ , and 1 cm. path) was kept between 0.200 and 0.400. In all media strictly exponential growth is maintained until the optical density reaches 0.800 or more. This optical density corresponds to $1.2-6.2 \times 10^8$ bacteria/ml. and to c. 140 μ g./ml. bacterial dry weight in the range of culture media employed.

Continuous culture growth. A continuous culture device using an automatic pipetting machine as feeding pump was employed (Formal, Baron & Spilman, 1956). The culture volume of 600 ml. contained in a cylinder 6 cm. in diameter, was aerated through a fritted glass plate. Efficient stirring was produced by the vigorous aeration; thus an added drop of dye solution became uniformly mixed in the culture liquid well within 1 sec. Excess fluid was continuously removed from the surface by suction. The dilution rate *D* (Monod, 1950) was varied by adjusting both the numbers of strokes delivered by the pipetting machine (10-15/min.) and the volume of medium added/stroke (0.5-1.5 ml.). Mutant *try A-8* was grown with tryptophan as limiting factor in the casamino-acids medium with 1 μ g. tryptophan/ml. Feeding was routinely started just after the culture had exhausted the tryptophan present at the time of inoculation. Sampling for the various analyses was carried out after

Table 1. *Culture media employed*

No.	Medium	Concentration	Average growth rate in	
			No. of doublings/ expt.	hr.
1	Brain + heart infusion	Full strength	1	2.80
2	Nutrient broth	Meat extract + 1 % peptone	3	2.75
3	Yeast extract + glucose	Full strength + 0.2 % glucose	2	2.73
4	Placenta broth	Full strength	1	2.70
5	Nutrient broth	Dil. 1:2 with medium no. 14	3	2.60
6	Nutrient broth	Dil. 1:5 with medium no. 14	9	2.40
7	Casamino acids ^(a)	1.5 % (Difco) + 0.01 % tryptophan in medium no. 14	2	2.00
8	199 Tissue-culture medium	See ^(b)	1	1.88
9	20 amino acids	As in medium No. 8 + salt solution ^(c)	1	1.83
10	Amino acids pool 2 ^(d)	As in medium No. 8 + salt solution ^(c)	2	1.46
11	Amino acids pool 3 ^(e)	As in medium No. 8 + salt solution ^(c)	2	1.38
12	Amino acids pool 4 ^(f)	As in medium No. 8 + salt solution ^(c)	1	1.25
13	Amino acids pool 1 ^(g)	As in medium No. 8 + salt solution ^(c)	1	1.22
14	Glucose salt (medium K)	0.2 % + Salt solution ^(c)	9	1.20
15	Succinate salt	0.2 % + Salt solution ^(c)	2	0.94
16	Lactate salt	0.2 % + Salt solution ^(c)	2	0.90
17	Dulcitol salt	0.05 % + Salt solution ^(c)	1	0.83
18	Aspartate salt	0.012 % + Salt solution ^(c)	1	0.83
19	Methionine salt	0.06 % + Salt solution ^(c)	1	0.81
20	Histidine salt	0.04 % + Salt solution ^(c)	1	0.78
21	Threonine salt	0.012 % + Salt solution ^(c)	1	0.63
22	Lysine salt	0.014 % + Salt solution ^(c)	1	0.62

(a) This medium, with limiting tryptophan, was employed in the bactostat experiments.

(b) Morgan's medium (Salk, Youngner & Ward, 1954) was employed without antibiotics, indicator and solutions H-K, I, J, Q, G and P.

(c) Salt solution: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; citric acid, 1.0; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 5.0; $\text{Na}(\text{NH}_4)\text{HPO}_4 \cdot 4\text{H}_2\text{O}$, 1.74; KCl, 0.74 g./l. Made up as a 50 × concentrate. This solution did not support perceptible growth without the addition of other carbon sources.

(d) Threonine, tyrosine, cysteine, histidine, phenylalanine, isoleucine, hydroxyproline and arginine.

(e) Phenylalanine, isoleucine, hydroxyproline, arginine, leucine, aspartic acid, glycine and tryptophan.

(f) Leucine, aspartic acid, glycine, tryptophan, glutamic acid, alanine, serine and valine.

(g) Glutamic acid, alanine, serine, valine, glutamine, lysine, methionine and proline.

not less than 6 hr. of growth. During this period the optical density remained practically constant at about 0.400.

Mass determination. The values of mass/cell are expressed as the optical density at 450 mμ (1 cm. path) given by a suspension containing 10^7 cells/ml. The optical density was found to be proportional to the dry weight, irrespective of the cell size. Optical density 0.100 corresponds to 17–18 μg. dry weight/ml.

Plate counts. Samples of the cultures were diluted in steps representing a total dilution of 2 or 4×10^{-4} . The original sampling was done with a 0.025 or 0.050 ml. constriction pipette and the subsequent steps were carried out with

0.1 ml. serological pipettes. For each value of Fig. 1 plating was done from at least six individual dilutions performed within 10 min. and adjusted to give between 300 and 600 colonies/plate. The viable counts were fitted to the growth curve determined by the optical-density measurements. It was found that the number of viable cells/ml. could be measured with an error of less than 10 %.

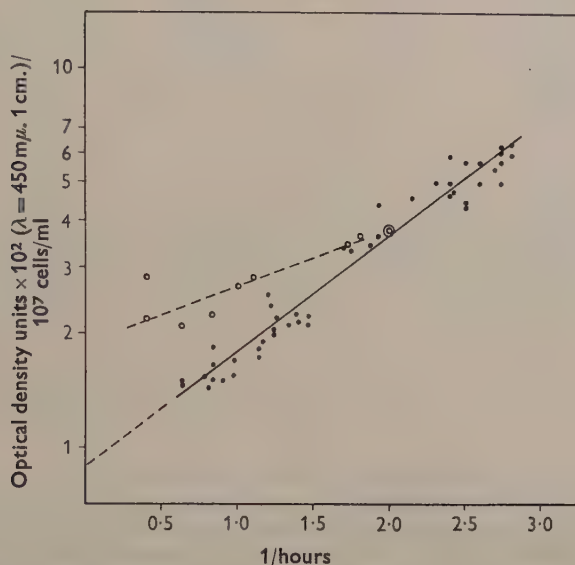


Fig. 1. Dependency of cell mass on growth rate at 37°. From the optical-density (mass) measurements and the viable counts in the different media, values for optical density/ 10^7 cells/ml. were calculated. The logarithm of these values is plotted against the growth rate expressed as doublings/hr. (●). The stippled line corresponding to the open rings and to the double-ringed point represents results from continuous culture experiments plotted against the dilution rate (○).

Chemical analysis. For nucleic-acid determinations, 40 ml. samples were frozen quickly in a solid CO_2 + ethanol bath. They were thawed and centrifuged in a cooled Servall angle centrifuge at 12,000 rev./min. for 20 min., the sediments were resuspended in 2.5 ml. of cold saline and 0.1 ml. of 70 % perchloric acid was added to 2 ml. of this suspension. The material was heated to 70° for 30 min., centrifuged and the supernate collected for colorimetric sugar tests. Deoxy-ribose was determined by the procedure of Burton (1956) on 1.0 ml. of the acid extract. Ribose determinations following the method of Kerr & Serai-darian (1945) were performed on 0.1 ml. of the extract. All spectrophotometry was done with a Zeiss Model PMQ II spectrophotometer employing 1 cm. cuvettes. Most of the values presented in Figs. 2 and 3 are averages of four independent determinations.

Nuclear staining. Fixation with OsO_4 and staining with thionine were carried out according to Lark, Maaløe & Rostock (1955), except that acid hydrolysis was extended to 6 min. This procedure is not primarily intended to preserve fine structural detail but, in the organism used, it reveals the same

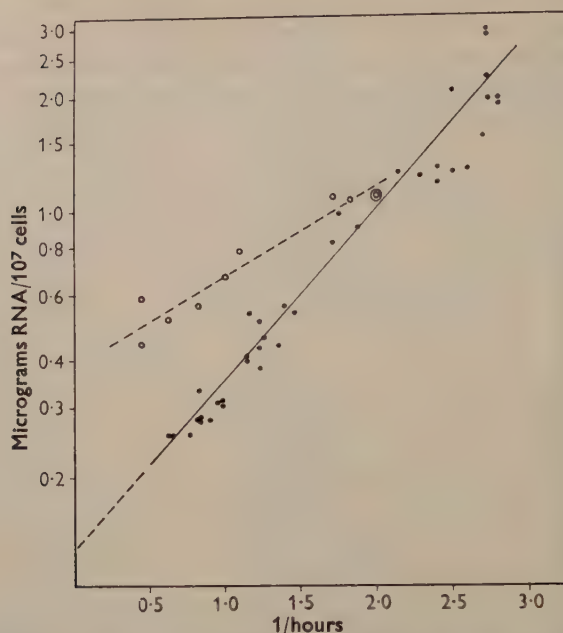


Fig. 2. Dependency of cellular ribonucleic acid on growth rate at 37°. The RNA content of the cultures was calculated from the ribose determinations. ($\mu\text{g. RNA} = \mu\text{g. ribose} \times 4.91$). The logarithm of the RNA values (micrograms)/ 10^7 viable cells is plotted against the growth rate (●). The stippled line corresponding to the open rings and to the double-ringed point represents results from continuous culture experiments, plotted against the dilution rate (○).

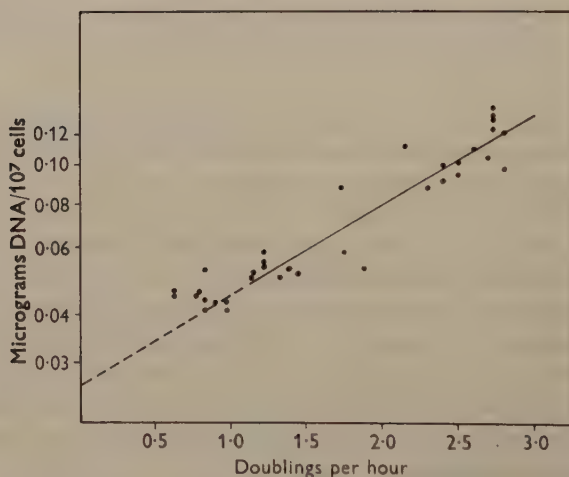


Fig. 3. Dependency of cellular deoxyribonucleic acid on growth rate at 37°. The DNA content of the cultures was calculated from the deoxyribose determinations ($\mu\text{g. DNA} = \mu\text{g. deoxyribose} \times 2.44$). The logarithm of the DNA values $\mu\text{g.}/10^7$ viable cells is plotted against the growth rate.

number of individual staining bodies in each cell as that obtained by other methods. For reasons of ease of observation the stained preparations were examined under phase contrast microscope (Zeiss). Between 300 and 400 cells per sample were scored as one-, two-, or four-nucleated taking into consideration, when possible, that some of the rods consisted of sister cells in different degrees of separation. A subjective criterion had to be employed in order to score a cell containing two adjacent bodies as one- or two-nucleated. However, repeated counts of the same preparation after an interval of months, or duplicate counts by different observers, always gave results compatible with the sampling error.

RESULTS

Balanced growth was maintained at 37° in the different media listed above (Table 1) and samples analysed for mass (optical density) RNA, DNA, viable counts and number of nuclei/cell. The results are presented in Figs. 1-3 in which logarithms of mass, RNA and DNA per viable cell are plotted against

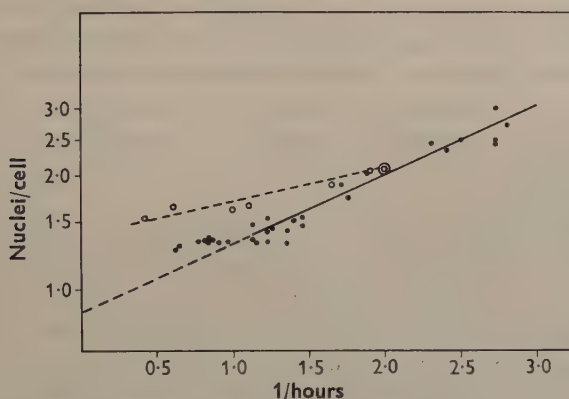


Fig. 4. Dependency of the number of nuclei/cell on growth rate at 37°. The average number of nuclei/cell was calculated from direct counts on stained preparations. The logarithm of the values is plotted against the corresponding growth rate (●). The stippled line corresponding to the open rings and the double-ringed point represents results from continuous culture experiments, plotted against the dilution rate (○).

the growth rate. Figure 4 represents a similar plot for nuclei/cell. Regression analysis showed that the straight lines drawn in Figs. 1-4 adequately represent the observed values. The individual determinations deviate only slightly more from the lines than is to be expected when the combined errors of the chemical analysis and the viable counts are taken into account. For ease of comparison, the increments on the logarithmic scale are the same in all figures.

In Fig. 1, the unbroken line fitted to the solid points shows that cell mass just about doubles/unit increase in doublings/hr. The extrapolation to zero growth rate suggests that the minimal bacterial size would be about half that of a cell growing in glucose-salt medium. This minimal size was actually attained under conditions of nitrogen starvation (see following paper).

Dr K. F. A. Ross kindly carried out measurements on the thickness of formalin-fixed organisms by interference microscopy using a modification of the technique he developed in 1955 (Ross, 1957). He obtained the following figures for the average of ten determinations per sample: 1.43μ for a culture growing in medium no. 2 at 2.73 doublings/hr. (d/hr.); 1.22μ for 1.85 d/hr. (medium no. 7); 0.93μ for 1.00 d/hr. (medium no. 14) and 0.87μ for 0.61 d/hr. (medium no. 22). The cell volumes obtained from these figures and from estimates of cell lengths were found to be proportional to the cell mass, as estimated from optical density measurements.

Figs. 2 and 3 show that, per unit increase in doublings/hr., the amount of RNA/cell increases by $\times 2.85$ that of DNA/cell by $\times 1.73$. The corresponding factor for the average number of nuclei/cell, derived from Fig. 4, is $\times 1.55$. At 37° , the four parameters examined may thus be described as exponential functions of the growth rate and can be arranged as follows, with regard to the slopes of the semilogarithmic plots: $\text{RNA} > \text{mass} > \text{DNA} \geq \text{nuclei/cell}$.

It is to be understood that all 'per cell values' are based, either on viable counts, or, in the case of nuclei, on cytological observations. Simultaneous haemocytometer and colony counts repeatedly showed that, under conditions of balanced growth, viable and total counts did not differ significantly. Counting unstained cells in the phase contrast microscope showed that, in all media, approximately 75 % of the units appeared to be true single cells, the remaining 25 % were 'doublets' representing incompletely separated sister cells. In Figs. 1-3, an approximate correction for doublets can be made by multiplying the ordinate values by 0.8.

The figures for nuclei/cell (Fig. 4) are based on direct counts of stained preparations. It was not always possible after staining to distinguish between single elements and doublets, particularly when the cells are small. Thus, at the lowest growth rates (smallest cells) uninucleated sister cells which remained attached to one another were probably often scored as binucleates. When the number of nuclei/cell is close to unity a bias of this kind may increase the observed value significantly over the true one. This might be the reason why, in Fig. 4, the experimental points deviate from the straight line at low rates, and it may also explain that the values for DNA/nucleus, which at higher growth rates are found to vary in a random manner around the mean value, decrease slightly, but significantly, in this region.

A number of experiments were carried out with cultures grown at lower temperatures. In Table 2 results obtained at 37° and at 25° with five different media are compared. In all cases, the growth rate at 25° was about half that at 37° ; nevertheless, mass, RNA, DNA and number of nuclei/cell remained nearly constant for a given medium. In broth or in the amino-acid medium the figures for nuclei/cell are somewhat higher at 37° than at 25° , but the experiments at 25° are few and the observed difference is probably not significant. Moreover, extensive chemical and cytological studies previously carried out with the same organism showed that, in broth, identical values for DNA and for nuclei/cell are obtained at 25° and at 37° (Lark & Maaløe, 1956; Lark, Maaløe & Rostock, 1955).

In fact, our data suggest that more extensive analyses of 25° cultures would permit graphs to be constructed which would be identical with those of Figs. 1-4 if the growth rate values on the abscissa were reduced to half. Thus, within the temperature range studied, *the size and chemical composition of the cells are related to the growth rate only in so far as it depends on the medium.*

Table 2. *The effect of temperature on cell size and composition*

Medium	No. of expts.	Growth temp. (° C.)	Doublings/hr.	Optical density* mass	RNA*	DNA*	Nuclei/cell
No. 6 (broth)	2	25	1.06	5.80	1.64	0.130	2.85
	4	37	2.40	5.00	1.44	0.095	2.50
No. 9 (amino acids)	1	25	0.88	3.66	0.92	0.085	2.05
	2	37	1.83	3.76	0.97	0.056	1.74
No. 14 (glucose)	3	25	0.65	2.32	0.56	0.065	1.46
	5	37	1.20	1.92	0.44	0.048	1.38
No. 15 (succinate)	2	25	0.48	1.47	0.39	0.038	1.31
	2	37	0.93	1.60	0.39	0.042	1.33
No. 16 (lactate)	2	25	0.50	1.50	0.39	0.038	1.30
	2	37	0.90	1.61	0.39	0.039	1.35

* The units for mass, RNA and DNA per cell are the same as in Figs. 1-3, respectively.

The results of the analysis of mass, RNA and nuclei/cell from the continuous culture experiments are presented as open circles in Figs. 1, 2 and 4. The logarithms of cell contents are plotted against the dilution rate D expressed in doublings of culture volume/hr. Monod (1950) and Novick & Szilard (1950) have shown that, in the ideal continuous culture system, D is related to the growth rate μ (in doublings/hr.) by the equation $\mu = D/\ln 2$. As will be seen, this situation was not obtained throughout our experiments. In Figs. 1, 2, and 4 the points marked with a double ring correspond to the maximum rate ($\mu = 2.0$) attainable during unrestricted growth of strain *tryA-8* in the Casamino acid medium with excess tryptophan. Thus, for values of D higher than 1.38, corresponding to $\mu = 2.0$, the theoretical growth rate calculated from the above formula exceeds the maximum values for unrestricted growth. D values as high as 1.77, corresponding to a theoretical growth rate of 2.6, have been obtained in our experiments. A similar discrepancy between the calculated and the maximum growth rates was reported by Herbert, Elsworth & Telling (1956) and Powell (1956) who attributed it to imperfect mixing.

It will be seen from Figs. 1, 2 and 4, that the values for mass, RNA and nuclei/cell for organisms grown in continuous culture may also be described as exponential functions of the growth rate. The slopes of the stippled lines drawn in Figs. 1, 2, and 4 are considerably less than those obtained for unrestricted growth, indicating that the lower the dilution rate, the more do the values exceed those from batch cultures. The same trend was observed for DNA, but the data are not included in Fig. 3 because of considerable scatter in the values obtained.

DISCUSSION

Our discussion falls into three parts: (1) An account of the physiological states imposed by the medium under unrestricted and restricted, balanced growth. (2) A representation of related and otherwise relevant data from the literature. (3) An analysis of our findings in terms of the major synthetic activities of the bacterial cell.

(1) In liquid cultures of low bacterial concentration, cells continue to grow for a long time in a virtually unchanging environment. This ideal condition leads to the establishment of a steady state of balanced growth, which can be prolonged at will by appropriate dilutions of the culture. In Campbell's apt formulation (Campbell, 1957), growth is said to be *balanced* over a time interval if, during the interval, every 'extensive' property of the system increases by the same factor. Failure to maintain balanced growth throughout an experiment makes it impossible to relate any measured quantity to the growth rate in a direct way.

When the density of a culture approaches saturation the growth rate gradually decreases and it increases upon subsequent dilution with fresh medium. In either case the changes in rates of cell division and of mass synthesis do not run parallel; as a rule, the rate of cell division remains unchanged for some time after the rate of mass synthesis has been lowered or raised in response to a change in the environment. This general phenomenon, which will be analysed in more detail in the next paper (Kjeldgaard, Maaløe & Schaechter, 1958), accounts for the well-known fact that 'exponentially growing cells' are bigger than 'resting cells'. These common, descriptive terms are sometimes taken to mean that only two physiological cell types exist: a small, resting cell and a larger, exponentially growing cell. This is, however, too simple a picture. Under conditions of *balanced growth*, one of a large number of possible physiological states is established. These states are characterized by the size and chemical composition of the cell; they depend on the culture medium, are not grossly influenced by the temperature of cultivation and can probably be maintained as long as the genetic stability of the culture permits. *At a given temperature*, size and composition are found to depend in a simple manner on the growth rate afforded by the medium. This implies that media which give identical growth rates produce identical physiological states, regardless of the actual constituents of the media. The 'resting state' finds a natural place in this system since, in an outgrown broth culture, the size of the cells is reduced to approximately the value expected for zero growth rate (see Fig. 1).

We have so far considered only batch cultures where all the relevant nutrients are present in excess in the medium. Because the growth rate is limited by the *type* of nutrients and not by their *concentration*, we refer to this situation as 'unrestricted growth'. We assume that the growth rate observed under these conditions is the highest which can be attained with the set of nutrients available to the cell. In a continuous culture device of the type we employed, growth is 'restricted' by the rate at which, say, a required

amino acid is added, i.e. by the low extra- and intracellular concentration of that component. Under conditions of unrestricted growth the concentration inside the cell of, say, an amino acid might similarly be thought of as being rate-limiting. This need not be the case, however, since, as discussed later, the growth rate may be controlled at the level of protein synthesis without involving limiting intracellular concentrations of amino acids, etc.

In our continuous culture experiments, new medium was added to the culture in pulses each of which momentarily increased the tryptophan concentration by 1–3 $\mu\text{g/l}$. According to Novick & Szilard (1950) a constant tryptophan concentration of about 3 $\mu\text{g/l}$. permits growth of *Escherichia coli*, strain B/1, *t* at maximum rate; but below this concentration the growth rate rapidly decreases. Assuming a similar concentration dependency for strain *tryA-8* of *Salmonella typhimurium*, every pulse of new medium will create conditions for growth at a relatively high rate for a short period. It is thus possible that, in our system, growth is intermittent, and that under such conditions, the growth rate during the pulse of growth afforded by a pulse of new medium, is not related to the dilution rate *D* in the simple manner proposed by Monod (1950) and Novick & Szilard (1950). We could therefore assume that the rate of synthesis during each pulse, rather than the overall rate, determines the size and chemical composition of the cell. However, experiments under more nearly ideal conditions of continuous growth are needed before the discrepancy between the results obtained under conditions of restricted and unrestricted growth can be properly analysed.

(2) Attempts to determine concurrently cell mass, nucleic acid content and the number of nuclei as functions of the growth rate have not previously been reported. Several pertinent studies exist in which one or more of these properties were related to the rate of growth. Thus, Wade (1952) obtained results which suggested a linear relation between RNA phosphorus/mg. N and the growth rate of *Escherichia coli*. Our data (Figs. 1, 2) indicate that this relation may actually be exponential; however, over the range studied by Wade and ourselves (0.6–2.8 generations/hr.), the increase in total RNA/unit mass (unlike RNA/cell) is at most twofold, and a clear distinction between linear and exponential functions therefore cannot be made. On the other hand, the overall increase is significantly less in Wade's experiments than in ours (50–60 % and 100 %, respectively). This difference may be due to the use by Wade of very large inocula (initial culture density: 2.5×10^8 to 10^9 organisms/ml.). According to our experience the cells of such a dense culture would not reach, or maintain, the size and RNA concentration characteristic of balanced growth. Wade's careful measurements show that the high growth rate could not always be maintained throughout the experiment; the RNA concentrations, which were related to the *initial* growth rates, may therefore be low compared to the values which would have been obtained had growth been truly balanced.

Caldwell & Hinshelwood (1950) and Caldwell, Mackor & Hinshelwood (1950) determined the RNA concentration (in mg. RNA-phosphorus/mg. N) in different strains of *Aerobacter aerogenes* under various conditions. From the

published data it cannot be ascertained whether balanced growth was attained, but their experiments also show that the faster the growth the higher the RNA concentration. In *Aerobacter*, the observed range of concentration is very high (three- to fourfold) perhaps because the lower growth rates were obtained by adding drugs to the medium or by using selected, slow-growing mutants.

Caldwell & Hinshelwood (1950) also determined the DNA phosphorus in various cultures of *Aerobacter aerogenes*. With increasing values for total nitrogen/cell, a slight, but probably significant rise in DNA/cell was observed. No comparison between these figures and the growth rates can be derived from their data.

Perret (1958), studying *Escherichia coli* strain K12, measured the mean cell length and the number of intracellular structures which appeared as transverse light bands under the phase microscope (probably equivalent to the nuclei described here). He also found that, to the different growth rates obtained in different media and in continuous cultures correspond definite values of cell length and number of 'nuclei'. His figures for nuclei/cell correspond closely to those of Fig. 4.

(3) The existence of a variety of stable physiological states cannot be explained in a simple manner. What follows is an attempt to analyse the significant features of our experimental findings in terms of the major synthetic activities of the cells.

Cytological evidence strongly suggests that the stained bodies referred to as 'nuclei' contain DNA, and we shall assume that most, if not all, the DNA of the cell is located there. A comparison of Figs. 3 and 4 shows that the amount of DNA per stained body is nearly constant, as would be expected if each body represents a nucleus in the physiological and genetic sense. With this in mind it seems opportune to differentiate between variation on the cellular and on the nuclear level. Since at low growth rates the majority of cells are uninucleated it seems natural to consider a single nucleus plus its corresponding cytoplasm, cell wall, etc., as an elementary unit. Multinucleated cells, composed of two or more such units can be thought of as syncytial. We will therefore distinguish between changes in the number of elementary units/cell and in mass/elementary unit. The way in which changes in the number of elementary units/cell come about is treated in the second paper of this series.

The variation in mass per elementary unit will be analysed below in accordance with the following assumptions: (a) that a large fraction of the cell's RNA is in the form of ribonucleoprotein particles consisting of about equal parts of RNA and protein and with molecular weight about one million (Schachman, Pardee & Stanier, 1952; Tissières & Watson, 1958); (b) that, per nucleus, the cell contains fixed amounts of DNA, cell wall and cell membrane material, varying amounts of free RNA and particles and, finally, a pool of soluble protein and other compounds; and (c) that this pool is always made up largely of protein.

The first assumption is supported by data from Wade & Morgan (1957). Comparing resting cells of *Escherichia coli* with cells growing in a complex medium these authors found that the RNA-pentose of particles sedimenting

completely in 4 hr. at 100,000 g amounted respectively to about 50 and 75 % of the total RNA-pentose. The second assumption is based on estimates of cell diameters and lengths (cf. p. 598) from which it can be estimated that the surface area/nucleus is virtually constant. The actual weight ascribed to cell wall and membrane (see Table 3) is of little importance for our conclusions. The third assumption seems more gratuitous; however, the greater part of the protein of the cell must be in the pool, and since this never exceeds about 60 % of the cell weight (Table 3, column 7) it is fair to assume that the bulk of the pool is protein (free enzymes).

Table 3. *Relative rates of protein synthesis*

1	2	3	4	5	6	7	8 Pool + particle protein synthesized/min.*	
Doublings/ hr.	Dry weight/ cell*	Nuclei/ cell	Dry weight/ nucleus*	RNA/ nucleus*	Particles/ nucleus†	Pool material/ nucleus*‡	Per nucleus	Per particle ($\times 10^4$)
0.6	240	1.25§	192	19	11,300	100	1.1	0.98
		1.10	218	22	13,300	120	1.3	1.0
1.2	360	1.45	250	31	22,400	135	3.1	1.4
2.4	840	2.40	350	65	54,000	176	8.8	1.6
2.8	1090	2.90	376	84	81,000	160	10.6	1.3

* All weights in g. $\times 10^{15}$.

† Calculated on the assumption that, from top to bottom, 50, 60, 70 and 80 % of the RNA is in particles of molecular weight one million, and composed of equal parts of protein and RNA.

‡ Per nucleus, the cell is assumed to consist of: 65×10^{-15} g. of wall, membrane and nuclear material, i.e. about one-third by weight of the smallest cell type; (b) particles and free RNA; and (c) a pool mainly containing soluble protein.

§ Two values given: above, the one directly observed; below, that obtained by extrapolating the linear part of the curve of Fig. 4 (see p. 597).

Table 3 shows representative figures for mass, average number of nuclei/cell and mass and RNA/nucleus, taken from Figs. 1, 2, and 4 (columns 2, 3, 4, 5). The right-hand side of the Table contains calculations based on these values and on the assumptions listed above. The number of particles and the pool size per nucleus are presented in columns 6 and 7. The relatively small changes in pool size show that variation in *mass/nucleus* is due mainly to variation in number of particles/nucleus.

In Table 3, column 8 presents maximum values for the synthesis of protein/nucleus/min. (assuming the pool to be all protein and adding to that the particle protein). It is apparent that the *rate* of protein synthesis is directly proportional to the *amount* of RNA, or the number of particles, and that both increase in rough proportion to the growth rate. Small variations in the soluble protein fraction of the pool or in the particle fraction of RNA do not seriously affect these trends. The rate of protein synthesis/unit RNA, or per particle, thus is nearly independent of the growth rate (column 9). This conclusion is of considerable interest because recent biochemical evidence indicates that these particles, which are analogous to the 'microsomal

particles' of animal cells, are directly concerned with protein synthesis (see, for example, review by Crick, 1958).

The constancy of the rate of protein synthesis/particle is most readily interpreted on the simple assumption that all particles participate equally in protein synthesis. This may, however, not be the case. By studying externally induced synthesis of β -galactosidase, it has been possible to show that the enzyme-forming system remains intact during growth in the absence of an inducer (Monod, Pappenheimer & Cohen-Bazire, 1952). If we tentatively identify the system producing β -galactosidase with one class of particles, we have a case where particles which continue to be reproduced remain virtually inactive unless an inducer is present. Addition to a culture of, say, an amino acid may cause repression of enzymes concerned with the synthesis of the added compound (see, for example, Vogel, 1957), and it is sometimes taken for granted that the process of repression also involves a reduction of the size of the enzyme-forming systems. If this is not true, as suggested by the results of Monod *et al.* (1952), cells growing in a complex medium would contain a certain fraction of inactive particles (corresponding to the repressed enzymes), whereas cells grown in minimal medium would contain predominantly active ones (little or no repression).

Without knowing what fraction of the particles is actively synthesizing, the true rate of protein synthesis/particle cannot be estimated. Despite this uncertainty it is clear that, unless a majority of the particles are rendered inactive during growth in complex media, the rate of protein synthesis per particle increases much less than does the growth rate.

It is attractive to imagine that the system responsible for a particularly complex process like protein synthesis perhaps functions with nearly the same efficiency under very different growth conditions (with the reservation, of course, that certain enzyme-forming systems may have *their* function specifically repressed). Addition to a culture growing in minimal medium of compounds like amino acids, purines or pyrimidines increases the growth rate presumably by relieving the cells of the necessity for synthesizing the added compounds. If one assumes that the economy of cell growth is actually based on maintaining a high efficiency of protein synthesis it is evident that an increase in growth rate is possible only if the protein synthesizing system of the individual cell is expanded; i.e. if the number of particles/nucleus is increased. For this to happen, the addition of, say, amino acids to the medium must cause a definite increase in the rate of RNA synthesis which, in turn, brings about the observed, *but smaller* increase in growth rate.

In the next paper we shall see that, in agreement with this hypothesis, the initial effect of adding amino acids or broth to a minimal medium culture is to stimulate RNA synthesis preferentially.

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The Transition Between Different Physiological States During Balanced Growth of *Salmonella typhimurium*

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SUMMARY: When cultures of *Salmonella typhimurium* undergoing balanced growth are shifted from one medium to another, a definite pattern of rate changes is observed. Shifts from a low to a high growth rate result in a strict succession of events: RNA synthesis is immediately affected and its rate rapidly increases to that characteristic of the new medium; the increase in optical density shows a lag of a few minutes before the new rate is attained; DNA synthesis and cell division, on the other hand, continue at the old rate for appreciable periods of time and then abruptly shift to the new rates. The times at which these shifts take place are, at 37°, invariably 20 and 70 min., regardless of the actual growth rates before and after the shift. This rate maintenance effect on DNA synthesis and cell division is discussed in terms of specific rate-controlling mechanisms.

In the preceding paper (Schaechter, Maaløe & Kjeldgaard, 1959) we showed that the size and chemical composition of *Salmonella typhimurium* vary exponentially with the growth rate afforded by different media. The present paper describes the transition from one state of balanced growth to another. The terms 'balanced growth' and 'cell' as used by us, have been defined in the preceding paper. Two types of experiments were performed, both involving precisely timed changes of medium at constant temperatures; one going to a higher growth rate (*shift up*), and the other to a lower rate (*shift down*).

The *shift up*, exemplified by going from a glucose salt medium to nutrient broth, is characterized by an orderly dissociation of the main synthetic activities. Upon addition of broth, the rates of synthesis of ribonucleic acid (RNA) and of total mass immediately increase, whereas deoxyribonucleic acid (DNA) synthesis and cell division continue *at the old rate* for considerable periods of time. The new state of balanced growth is reached when, after about 70 min. (at 37°), the rate of cell division abruptly changes from the pre-shift value to the definitive broth rate. We interpret this 'rate maintenance effect' as evidence for the existence of separate rate-controlling mechanisms.

The *shift down* is rapidly effected by filtration of, say, a nutrient broth culture and resuspension of the cells in simple defined medium. It is characterized by a period during which cell division and DNA synthesis continue in the absence of net synthesis of RNA and total mass. By the time RNA and mass begin to increase the cells have already approached the size and the composition typical of balanced growth in the simpler medium. This behaviour

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demonstrates that certain synthetic abilities are repressed during growth in a rich medium and that it takes considerable time to re-establish all the functions necessary for growth in the poorer medium.

METHODS

Strains. *Salmonella typhimurium*, see Schaechter *et al.* (1958). *Escherichia coli*, strain K 12 (58-161), methionine-requiring, was obtained from Dr W. Hayes. *E. coli* strain ML37, an inducible β -galactosidase former, was obtained from Dr J. Monod.

Media. See Schaechter *et al.* (1958).

General procedures

Shift up: to a culture grown for several generations under conditions of balanced growth was added an equal volume of double-strength medium supporting faster growth.

Shift down: a culture maintained under conditions of balanced growth for several generations was rapidly filtered on a membrane filter (Membranfilter-Gesellschaft, Sartorius-Werke A. G., Göttingen). The filter was washed with portions of the new medium, the bacteria shaken off and resuspended. This operation usually took 2-3 min. All fresh media were pre-warmed to 37°.

Conditions of aeration, bacterial density, chemical analysis, nuclear staining, colony counts, etc., were described in Schaechter *et al.* (1958).

RESULTS

A typical *shift up* at 37° between minimal medium with glucose as energy source (average generation time: 50 min.) and nutrient broth (average generation time: 22 min.) is illustrated in Fig. 1 for *Salmonella typhimurium*. After addition of broth a characteristic dissociation of the main synthetic activities was observed, as follows. (1) RNA synthesis was immediately and strongly stimulated and, for a short period, took place at a rate *higher* than that characteristic for broth. (2) Mass synthesis (as inferred from optical density measurements) rapidly increased and, after about 5 min., reached the rate typical of balanced growth in broth. (3) DNA synthesis remained unaffected for about 20 min., at which time its rate suddenly changed from the pre-shift value to the definitive broth value. (4) The average number of nuclei/cell stayed constant at about 1.5 until about 35 min. after the shift, and then, within 15-20 min., increased to 3.0. (5) Cell division, finally, continued at the pre-shift rate for about 70 min., when the rate suddenly increased and assumed the definitive broth value. The cells now reached the size and composition previously established for balanced growth of this organism in broth (see Schaechter *et al.* 1958).

The transition pattern illustrated in Fig. 1 has three distinguishing features: first, the order in which the synthetic activities respond to the addition of broth; secondly, the precise maintenance of the pre-shift rates of DNA synthesis and cell division for about 20 and 70 min., respectively; and thirdly,

the abruptness with which, at these times, the definitive broth rates are established.

These characteristics are reproduced with remarkable constancy with related organisms. In a glucose salt medium (with methionine added) and in broth *Escherichia coli*, strain K12 (58-161), exhibited growth rates similar to those obtained with *Salmonella typhimurium*, and the transition pattern of Fig. 1 was almost duplicated. Strain ML37 of *E. coli* was tested in a shift

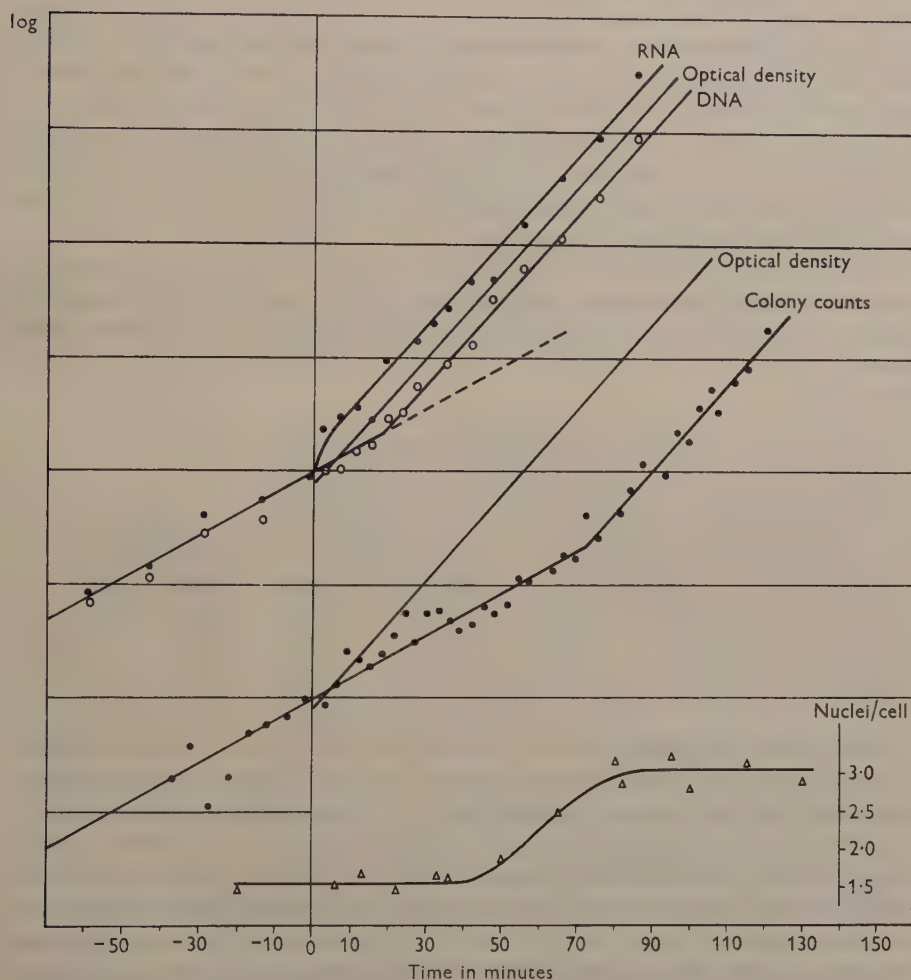


Fig. 1. *Salmonella typhimurium* culture at 37°, shifted from glucose salt minimal medium to broth at time 0. Optical density, viable counts, RNA and DNA content were frequently determined. For the sake of clarity two separate sets of curves are presented: on top, optical density, RNA and DNA, and below, optical density and viable counts. In all cases the logarithms of the measured values are plotted against time and all values are transposed so as to make the curves representing balanced growth in minimal medium coincide. The distance between horizontal lines corresponds to one doubling. In lower right corner the average number of nuclei/cell obtained from direct counts on stained preparations are plotted against time.

between a succinate salt medium and broth; in this case the final growth rate in broth (average generation time: 22 min.) was more than three times the pre-shift rate (average generation time: 69 min.) but, again, an abrupt change from the low to the high rate of cell division occurred about 70 min. after the shift.

Several *shifts up* were performed with *Salmonella typhimurium* between different pairs of media. Media with amino acids, casein hydrolysate or the more complete defined medium 199 were substituted for broth in shifts from the usual glucose-salt medium; in other experiments, pre-shift growth rates as low as 0.6 doublings/hr. were obtained by using single amino acids or rhamnose as carbon and energy sources instead of glucose. In all cases the transition pattern of Fig. 1 was observed and the changes in the rates of DNA synthesis and cell division always occurred about 20 and 70 min., respectively, after the shift. Thus, *at a given temperature, the time during which the low, pre-shift rates were maintained was independent of the absolute growth rates in the media between which the shift was made.*

Experiments done at lower temperatures with *Salmonella typhimurium* showed the usual dissociation pattern, but with a change in the time scale. Shifts were carried out at 30° and 25° between the glucose salt medium and broth; the results are presented in Table 1 together with data from a 37° experiment.

Table 1. *Shifts of Salmonella typhimurium from glucose salts medium to broth at different temperatures*

Temp.	Generations/hr.		Time of change (min.) in rate of		Factor of increase in	
			DNA synthesis	Cell division	DNA/ cell	Mass/ cell
	Pre-shift	Final				
37°	1.20	2.40	25	75	2.0	2.6
30°	0.73	1.75	—	90	—	2.5
25°	0.62	1.15	60	150	1.8	2.3

In the preceding paper we demonstrated that, during balanced growth, the size and composition of *Salmonella typhimurium* cells depend almost entirely on the medium and not on temperature. In all the media tested the growth rate at 37° was about twice that obtained at 25°. The figures of Table 1 show that, like the generation time, the periods during which the pre-shift rates of DNA synthesis and of cell division are maintained are roughly doubled when the temperature is reduced from 37° to 25°. In fact, our experiments indicate that the set of curves of Fig. 1 can be used to illustrate shift experiments carried out at different temperatures simply by adjusting the time scale. For instance, 1 hr. at 37° is equivalent, in all respects, to about 2 hr. at 25°.

In a *shift down* the dissociation pattern differs in several ways from that of a *shift up*. Fig. 2 shows a typical experiment in which cells from a broth culture of *Salmonella typhimurium* were collected by filtration, washed on the filter and resuspended in glucose salt medium. Immediately after transfer, net synthesis of mass and RNA stopped for 30–40 min. (indeed, the amount of

RNA usually dropped slightly). During this period, cell division and DNA synthesis continued until the cell number had increased by about a factor of two and the amount of DNA somewhat less. As a result the average number of nuclei/cell was reduced; in two experiments it decreased from 2.4 to 1.6 and from 2.5 to 1.7, respectively. The dissociation of the synthetic activities observed during the first 30–40 min. after the *shift down* achieved a partial reversion to the size and composition characteristic of cells growing in minimal

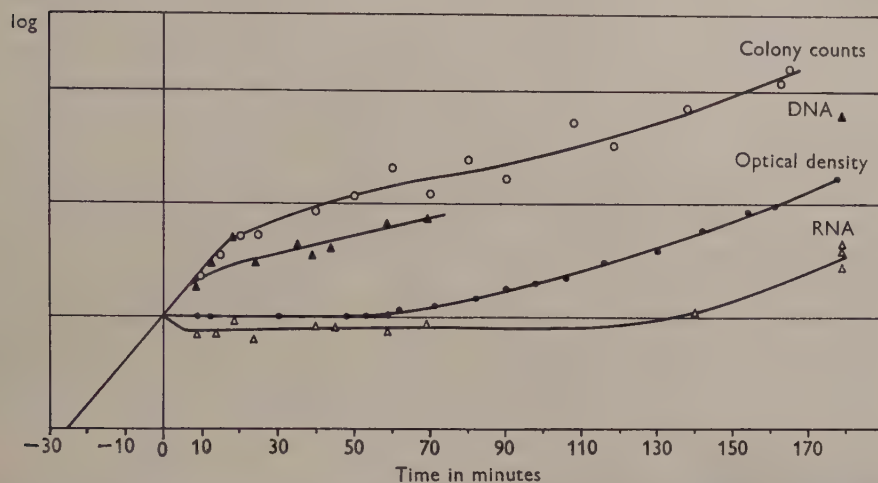


Fig. 2. *Salmonella typhimurium* culture at 37° shifted from broth to glucose salt minimal medium at time 0. Optical density, viable counts, RNA and DNA content were frequently determined. The logarithms of the measured values are plotted against time and all values are transposed so as to make the curves representing balanced growth in broth coincide. The distance between horizontal lines corresponds to one doubling.

medium. Complete reversion, however, took several hours, during which time the rates of synthesis of RNA and of cell mass gradually increased until the minimal medium values were reached.

Parallel platings on broth and on minimal agar gave identical colony counts; this showed that the lag in RNA and mass increase following a *shift down* was not, even in part, caused by loss of viability.

Several authors have shown that addition to a minimal medium culture of, for example, an amino acid, results in repression of the production of enzymes concerned with the synthesis of this compound (see, for example, Vogel, 1957). It may be assumed that, during growth in broth, a large number of enzymes are repressed. The kinetics of this 'collective repression' of enzyme synthesis was studied by carrying out first a *shift up* (from glucose minimal medium to broth) and then, at various times thereafter, a *shift down* (back to glucose minimal medium). The optical density was frequently measured in all the cultures and, after each *shift down*, the culture was followed until the definitive rate of mass increase had been established. A plot of the logarithm of the optical densities versus time is presented in Fig. 3. The transfer of the original minimal medium culture to broth took place at time 0 and the steep

line represents the growth of the broth culture. The times of return to minimal glucose medium are marked on this line by arrows. The figure shows that the first 10–15 min. in broth do not affect the ability of the cells to resume growth at optimal rate in minimal medium. Thereafter, an increasing lag is observed until, after about 150 min. in broth (i.e. approximately a 64-fold increase in bacterial mass), it reaches a maximum value. All cultures retransferred to

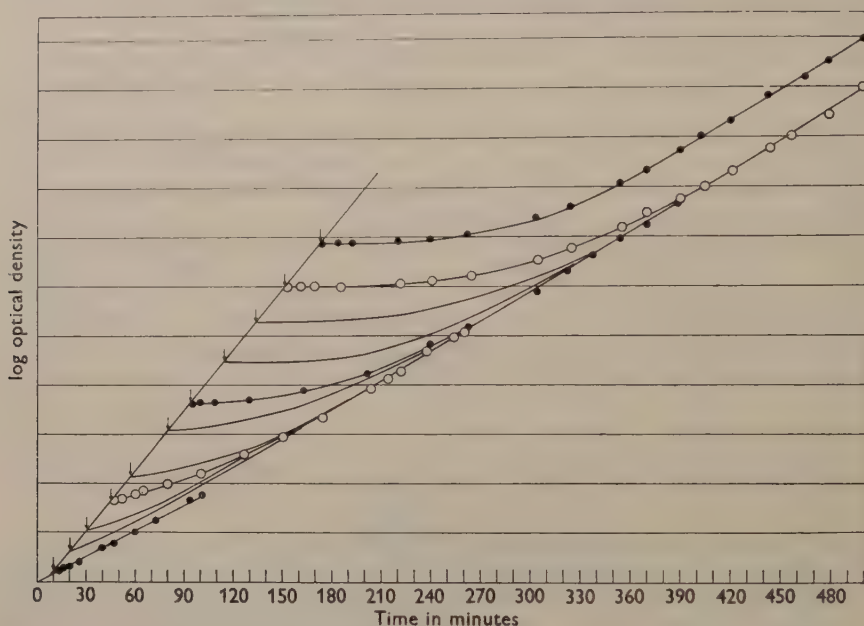


Fig. 3. A glucose salt minimal medium culture of *Salmonella typhimurium* at 37° was diluted with broth at time 0 and the optical density of the culture followed. At various times after the shift samples of the culture were returned to glucose salt minimal medium and the optical density followed. The logarithms of the optical densities are plotted against time. The steep linear curve represents the growth curve of the broth culture and the arrows mark the time of return to minimal medium. The optical-density curves of the minimal medium cultures are transposed so as to obtain continuity with the broth curve at the time of transfer. For reason of clarity experimental points are not shown on all curves. The distance between horizontal lines corresponds to one doubling.

minimal medium between 15 and 150 min. after the *shift up* ultimately follow the same growth curve. This indicates that during this time the length of the lag, or recovery period, is proportional to the time of growth in broth minus 15 min.

Finally, *shifts down* were carried out between the complete glucose salt medium and the same medium without a nitrogen source. As in the experiments described above, cell division continued after the shift in the absence of net increase in cell mass, until the average cell size had been reduced to half. The cell size attained corresponds fairly well to the value obtained by extrapolating to zero growth rate in Fig. 1 of the preceding paper. No loss of viability seemed to occur during the first 3 hr. after the shift to minimal medium without

nitrogen source. This was indicated by the fact that, upon addition of a nitrogen source or broth, the optical density of the culture immediately started increasing as in an ordinary *shift up*.

DISCUSSION

In the preceding paper (Schaechter *et al.* 1958) we showed that, under conditions of unrestricted balanced growth, mass, RNA and DNA/cell change exponentially with the growth rate. The transition from one state of balanced growth to another therefore requires a period of adjustment, the characteristics of which depend on the direction of the shift.

Shifts with rate increase (*shift up*) show a remarkably constant pattern under various experimental conditions. With different strains and at different temperatures it is uniformly found that the low, pre-shift rates of cell division and of DNA synthesis are maintained for well-defined periods, whereas the rates of mass increase and of RNA synthesis rapidly change to higher values (see Fig. 1 and Table 1).

This pattern is strikingly different from that observed when raising the temperature of a broth culture from 25° to 37° (Lark & Maaløe, 1956). In this case the low, 25°, rate of division was maintained for about 20 min., but the amount of DNA/cell almost doubled during the first 5–10 min. after raising the temperature. This burst of synthesis was followed by a period with no net increase in DNA and a second, but smaller, burst of synthesis. In the temperature shift, which involves no overt change in the definitive size and composition of the cells, the rate of DNA synthesis thus cycles between extremely high and low values before the final 37° rate is established.

On the basis of diffusion considerations Rashevsky (1948) suggested that division might be the direct consequence of cells reaching a critical size dependent on the physiological state. If this mechanism applied to *Salmonella typhimurium*, division would be expected to stop completely after a *shift up*, until the cells which were about to divide at the time of the shift had reached the critical size characteristic of the new medium. Fig. 1 shows that division does not stop even for a short time but continues at the pre-shift rate for a very considerable period. The mechanism responsible for this *rate maintenance effect* is unknown and no simple model can be suggested.

In the case of DNA synthesis the period preceding the rate change is only about 20 min. and it is difficult to decide from individual experiments whether the pre-shift rate is strictly maintained. However, the cumulated evidence from many experiments shows that no lag is produced and that the rate of synthesis during this 20 min. period is very close to the pre-shift rate. Like the synthesis of cell wall material (the general term 'wall material' is used here because we lack precise knowledge about the components of the cell wall and/or membrane, which are directly involved in the division process), the synthesis of DNA thus seems to be governed by a rate-controlling mechanism which, for a definite period, counteracts the metabolic factors responsible for the rapid increase in the rates of RNA and protein synthesis after a *shift up*.

The two control mechanisms have characteristic common features: First, at a given temperature, the periods during which the low, pre-shift rates are maintained are constant and independent of the absolute growth rates in the two media. These periods therefore bear no simple relation to either the pre- or the post-shift generation times. Secondly, if, between two temperatures the generation time doubles the control periods also double (Table 1). Thirdly, when these controls are eventually overcome, the new and higher rates are rapidly established, irrespective of the difference between pre- and post-shift rates. On the other hand, the two mechanisms are entirely separable since, after a temperature shift, the control of DNA synthesis breaks down at once, that of cell division only after about 20 min. at 37° (Lark & Maaløe, 1956).

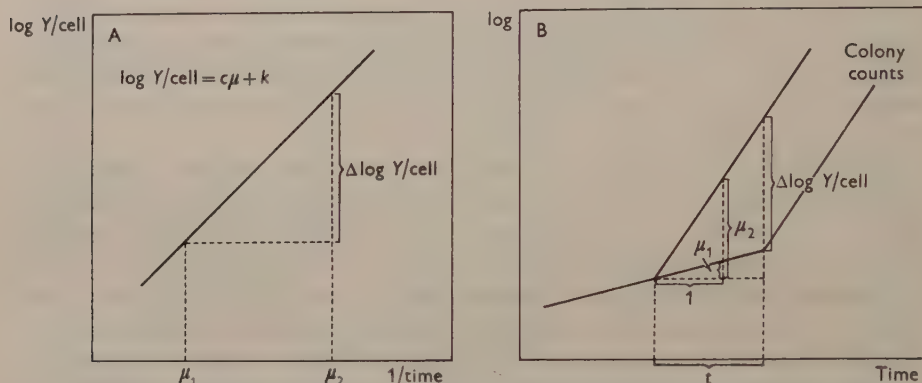


Fig. 4. Schematic illustration of the plot of cell content versus growth rate (A) (see preceding paper) and of a *shift up* experiment (B). μ_1 and μ_2 represent the rates before and after the shift, respectively. Y is the concentration of a given cell constituent. From the linear curve with slope c in graph A it is seen that the increase in the logarithm of Y/cell ($\Delta \log Y/\text{cell}$) for a rate change from μ_1 to μ_2 equals $c(\mu_2 - \mu_1)$. From graph B, where μ_1 and μ_2 are the slopes of the growth curves and t the time between rate changes, it follows that $\Delta \log Y/\text{cell} = t(\mu_2 - \mu_1)$. Consequently, for any μ_1 and μ_2 , t equals c (see text).

In the intact cell, DNA synthesis presumably takes place in the 'nuclear regions', and it is natural to imagine that the system responsible for the synthesis of wall material forms part of the cell wall itself or of the cell membrane. The two functions therefore seem to be separated physiologically as well as anatomically from the cytoplasm. Within the cytoplasm the small-particle fraction, which seems to contain most of the RNA (Schachman, Pardee & Stanier, 1952), may constitute a third system, distinguishable by being particularly sensitive to changes in the medium. As shown in Fig. 1, RNA synthesis is unique in the sense that, in a *shift up*, the initial rate exceeds the definitive one.

The strict time schedule for the rate changes which is suggested by our experiments can also be deduced from the exponential relations between growth rate and cell mass and DNA content previously established (see Fig. 4 and legend). Assuming that the transition pattern regularly observed in

our experiments holds for shifts between any pair of media, theoretical times for DNA and colony count rate-changes were calculated from the slopes of the semi-logarithmic plots of cell mass and cell DNA versus growth rate (Figs. 1 and 3 in preceding paper). Taking into account that the optical-density curve has a lag of about 5 min., the theoretical times for changes in rate after the medium shift (at 37°) are: 18 min. for DNA, and 66 min. for colony counts. Both values are in good agreement with the experimental findings (about 20 and 70 min.)

The classical representation of the growth curve begins with a lag phase and a period of increasing growth rate which leads into the phase of exponential growth. Often, an outgrown culture contains a large percentage of non-viable cells whose presence veils the true mass increase of the viable bacteria and creates an apparent lag in the optical-density curve. On the assumption that all viable cells in such a mixed population start growing at maximum rate, an estimate of the fraction of non-viable cells was obtained by extrapolating the linear part of the growth curve, as determined by optical-density measurements, to zero time. This estimate was found to be in close agreement with the results we obtained by comparing viable and total cell counts.

It therefore looks as if all viable bacteria after transfer to a new medium start almost immediately to increase in mass at maximum rate, as has already been suggested by Hershey (1938). Colony counts, on the other hand, remain constant for about 70 min. (maintenance of zero rate), before changing suddenly to the maximal rate. From the results of similar experiments, published by Lark & Maaløe (1956), a lag period for DNA synthesis of about 20 min. can be estimated. The viable cells of an outgrown broth culture can be shown to possess a more complete spectrum of enzymic activities than the exponentially growing 'broth cells'. Thus, when *shifts down* (to glucose salt medium) were carried out at various times during the transition from the exponential to the 'resting' phase of a broth culture, we observed that the lag in mass synthesis normally following such a shift became progressively shorter as the broth culture approached the resting stage. During the decline phase of a broth culture, the cells therefore seem gradually to regenerate their full synthetic capacity. To support this point we have measured the β -galactosidase in broth-growing cells of a constitutive strain of *Escherichia coli* (ML308). It was found that during the exponential phase enzyme synthesis was repressed and that, as the culture passed into the decline phase repression was released, with the result that the amount of enzyme/cell increased considerably.

The experiment of Fig. 3 is compatible with the assumption that, after a *shift up*, the overall activity of the repressed enzymes is lost by dilution. This has already been shown to be the case for a number of inducible enzymes (see review by Cohn, 1957).

The dependency of cell size and overall chemical composition on the growth rate described by Schaechter *et al.* (1958), and the transition pattern discussed above lead to a few general conclusions and invite much speculation.

The results of the shift experiments indicate that two of the major synthetic functions of the bacterial cell form separate systems within which different, distinct control mechanisms operate. Thus, on the background of cytoplasmic

protein and RNA synthesis, the rates of which almost immediately respond to changes of medium or temperature, the synthesis of DNA and of cell wall material stand out as individual, autonomous functions.

It may be long before the mechanisms invoked to account for the transition pattern can be discussed in specific chemical terms. Speculations in this direction would seem premature. We want to stress again, however, that control mechanisms with the properties described above would suffice to account quantitatively for the observation that cell size and composition are determined uniquely by the growth rate afforded by the medium.

The evolutionary significance of our observations is difficult, if not impossible to assess. Superficially, it seems a paradox that the *fewer* the synthetic activities required during growth the *bigger* the cell. On the other hand, if the hypothesis advanced by Schaechter *et al.* (1958) is correct, the growth rate cannot increase upon transfer to a richer medium unless the protein synthesizing system first expands. In other words, the cell must grow bigger, and increase its RNA/nucleus ratio, in order to grow faster; without this adjustment growth in the richer medium would be unbalanced. In *Salmonella typhimurium* the necessary enrichment in RNA is effected through the rate control, which delays DNA synthesis by maintaining the old rate for about 20 min. after a *shift up*. In a sense, it may thus be said that the control mechanism enables the cells eventually to grow faster in the new medium, and thereby confers a selective advantage upon them. It should be borne in mind, however, that the control mechanism might conceivably be an integral and indispensable part of the DNA synthesizing system; if so, it makes no sense to talk about selective advantage in this connexion.

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The Nutritional Requirements of Rumen Strains of *Streptococcus bovis* Considered in Relation to Dextran Synthesis from Sucrose

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SUMMARY: Five rumen strains of *Streptococcus bovis* all required biotin for good anaerobic production of dextran from sucrose in a simple acetate + bicarbonate + amino acids medium. Three of these strains required at least pantothenate also. When phosphate replaced acetate as a buffer, dextran production did not occur unless a soluble protein was also supplied in the medium.

Ford, Perry & Briggs (1958) recently showed that not a single strain among 26 rumen strains of *Streptococcus bovis* required any B-vitamins for anaerobic growth in a glucose medium although, in agreement with previous observations of Niven, Washburn & White (1948), they found that some of these strains appeared to require one or more B-vitamins when incubated aerobically. In view of the observations of Bailey & Oxford (1958*a, b*), who showed that all of 16 rumen strains of *S. bovis* could produce good yields of dextran in a complex sucrose liquid medium only when certain cultural conditions were provided, it became of interest to determine whether these strains could ever produce dextran in a simplified protein-free medium devoid of all, or most, B-vitamins. The cultural conditions necessary for dextran production at 37° were the provision of ample carbon dioxide and of a high concentration of phosphate or acetate. The nutrient basal medium used by Bailey & Oxford was, however, complex, containing yeast extract and Casitone as sources of B-vitamins and organic nitrogen. With such a medium, strict anaerobiosis was neither necessary for, nor detrimental to, dextran production. The enzyme responsible for dextran production, dextransucrase, is secreted into the medium, and might conceivably need the provision of a large stabilizing colloid molecule for efficient action at 37°, since it is known that the *Leuconostoc* dextransucrase, secreted at 25°, is not stable at 37° (Bailey, Barker, Bourne & Stacey, 1957). Nevertheless, it has proved possible to devise a simple medium for good dextran production by *S. bovis* at 37° which contains no molecules larger than an amino acid and an oligosaccharide. During this work some observations have been made on the apparent need for biotin and pantothenic acid for good dextran production by some strains of *S. bovis* even under anaerobic conditions.

METHODS

Rumen strains of Streptococcus bovis. Five isolates were used of which three (2SA, 2B and 18/M2) had been isolated at the Rowett Research Institute, Aberdeenshire, before 1955, and the remaining two (I and 40) at this

laboratory quite recently (Bailey & Oxford, 1958*a*). Isolates 2SA and 40 had previously been shown to behave a little differently from the others, in that they produced small yields of dextran in absence of added CO₂.

Basal medium containing sucrose. The following was sterilized by intermittent steaming: Bacto-Casamino acids ('technical', 'certified' or 'vitamin-free' grades), 25 g.; Na thioglycollate, 0.3 g.; MgSO₄·7H₂O, 0.2 g.; FeSO₄·7H₂O, 0.005 g.; uracil, 0.02 g.; adenine sulphate, 0.05 g.; guanine, 0.02 g.; cystine and tryptophan, each 0.15 g. dissolved in a little boiling dilute sulphuric acid; Analar sucrose, 50 g.; distilled water, 1000 ml.; 40 % (w/v) KOH solution, a few drops to bring to pH 6.

Additions to basal medium. Crystalline sodium acetate (2 g./100 ml.) or Na₂HPO₄·12H₂O (1.8 g./100 ml.) to serve as buffering agents were usually sterilized separately in the solid state in plugged 50 or 100 ml. conical flasks in which the cultures were afterwards to be grown. Alternatively, acetate (2 %) but not phosphate could be added to the basal medium before sterilization. The following sterile solutions were also added to the flasks containing buffer salts after their sterilization: 5 % (w/v) K₂CO₃ (0.6/100 ml.); B-vitamin solution (1/100 ml.). The flasks were then filled almost full with the basal medium and the contents well mixed by a sterile glass rod. The vitamin solution contained one or more of the following eight ingredients: pyridoxine HCl, nicotinic acid, calcium pantothenate, thiamine HCl, *p*-aminobenzoic acid (each 200 mg./l.); riboflavin and folic acid (each 50 mg./l.); and biotin (0.25 mg./l.). Another adjuvant used in conjunction with phosphate buffer only was 3 % (w/v) Bacto-tryptose before and after dialysis for 24 hr. at 2°. This solution was then suitably diluted and used in place of distilled water in making up the basal medium containing sucrose. The final concentration of Bacto-tryptose, or its dialysed equivalent, varied from 0.3 to 1.5 % in this basal medium.

Cultural conditions. The primary inoculum for 50 ml. medium was one small loopful of a 1-day culture in Bacto-thioglycollate medium containing 1 % (w/v) sucrose. The temperature of incubation was 37°. Incubation was either in an almost 100 % CO₂ atmosphere (see Bailey & Oxford, 1958*a*) or in a metal McIntosh & Fildes' anaerobic jar filled with a 5 % (v/v) CO₂ + 95 % (v/v) H₂ gas mixture. A tube of sterile sucrose + Bacto-thioglycollate medium containing methylene blue was also placed in the jar to indicate whether anaerobic conditions had been maintained. After 1 day the cultures were removed from the jar and when growth or dextran production was not very pronounced they were incubated for a further day in air. The addition of K₂CO₃ to the medium was to ensure the presence of some dissolved CO₂, soon after lactic acid formation occurred, whatever gas mixture was above the culture.

Assessment of growth and dextran yield. When little or no dextran production occurred, growth was judged by the amount of deposit (±, + or ++) on centrifuging the culture clear. Dextran-producing cultures had first to be diluted with 2 vol. of water before centrifuging. Good dextran production (+ or ++) was revealed by: (a) a distinctly opalescent appearance; (b) considerable increase in viscosity; (c) difficulty in spinning down more than a

small fraction of the organisms in an undiluted culture; (d) immediate white precipitate on gentle shaking after adding ethanol (4 vol.) to the supernatant fluid from the undiluted culture (see Bailey & Oxford, 1958*a*). Trace amounts of dextran, requiring prolonged shaking for separation, were ignored. The final pH value of the culture was also roughly determined by means of B.D.H. 4055 indicator paper.

RESULTS

Acetate buffered medium

Effect of pantothenic acid in a simple amino acid medium not specially freed from B-vitamins. Most of the preliminary work was carried out with strain I, which had been chiefly used by Bailey & Oxford (1958*a, b*), and with a medium containing Bacto 'technical' or 'certified' grades of Casamino acids. The former contains more ash than the latter and there is no need to add ferrous sulphate to the medium when using it. Neither grade is guaranteed to be vitamin free; nevertheless, with use of either in conjunction with the usual procedure of omitting added B-vitamins one at a time from the total mixture of eight, it was clearly shown that good growth and dextran production took place with strain I only in those media containing added pantothenic acid. Likewise, also in a CO₂ atmosphere, growth and good dextran production took place when pantothenate, alone among B-vitamins, was added to the basal medium containing no other added vitamins. When, however, incubation was carried out in air, the growth was always very poor and no dextran resulted even when pantothenate was added. Incubation under strict anaerobic conditions gave moderate growth, with a decrease in pH to 5.2, but no dextran with no added pantothenate, although there was good growth (final pH 4.5) and good dextran production when pantothenate was added. Although therefore strain I is less nutritionally exacting anaerobically than in presence of oxygen (cf. Ford *et al.* 1958), pantothenate as well as CO₂ must always be supplied for good dextran production to occur. Presumably, the two grades of Casamino acids used are at least pantothenate-free, although adequately supplied with biotin (see below). The observation of Ford *et al.* (1958) was confirmed, namely, that pantothenate was not needed for good growth anaerobically when glucose was supplied in place of sucrose.

Repetition of these experiments with the other 4 strains showed that strains 18/M2 and 2B, but not strains 40 and 2SA, behaved as did strain I above. The abnormal strains seemed to have less need of pantothenic acid for dextran production than the others (see Table 1). Growth, however, was usually much slower without added pantothenate. Throughout this study good growth resulted also in a decrease in pH value to less than 5.0. Furthermore, there was never good dextran production without good growth.

Biotin as an essential for dextran production. It was realized from the above results that nothing more than a requirement of some strains of *Streptococcus bovis* for pantothenate for dextran production had so far been proved. This was confirmed by use of 'vitamin-free' Casamino acids. In addition, an absolute requirement for biotin for dextran production in all 5 strains came

to light. Thus, when to the basal medium were added the seven B-vitamins but not biotin, none of the 5 strains gave other than poor growth and no dextran under any conditions, including strict anaerobiosis. With all eight B-vitamins added, normal growth and good dextran production took place with all 5 strains, and good dextran production also occurred on the second transfer to this medium. When, however, biotin alone, or biotin + pantothenate only, were added to the basal medium, strain differences became apparent, and even as few as 5 strains fell into three distinct groups.

Thus isolates I, 18/M2 and 2B: poor growth and no dextran with biotin alone; good growth and dextran production with biotin + pantothenate.

Isolate 2SA: fair growth and dextran with biotin alone; good growth and dextran production with biotin + pantothenate.

Isolate 40: fair growth but no appreciable dextran with biotin + pantothenate; good growth and dextran production with all eight B-vitamins.

Furthermore, although growth could be quite good in a second serial transfer in the biotin + pantothenate medium, dextran production often failed to occur except in traces. For this reason it is always advisable to include the eight B-vitamins in the simplified medium when good yields of dextran are the objective. This behaviour recalls the variable behaviour towards B-vitamins of strains of *Streptococcus bovis* on aerobic incubation in glucose media (Ford *et al.* 1958). With sucrose media, however, it seemed to matter little whether incubation was under strictly anaerobic conditions or in nearly 100 % CO₂ containing a little air.

Phosphate buffered medium

Effect of dialysed tryptose on dextran production. All the following experiments were carried out in a CO₂ atmosphere with a basal medium containing 'technical' Casamino acids and with seven of the eight B-vitamins added, pantothenate being omitted. Whenever good growth took place in the acetate-buffered medium, there was almost always good dextran production, but the two phenomena seldom went hand in hand when phosphate was the buffering agent. From Table 1, which summarizes the results for all five isolates of *Streptococcus bovis*, it will be seen that only isolates 2SA and 40 gave good dextran production in the phosphate medium, although all 5 strains grew quite well. Furthermore, addition of pantothenate seemed not to be advantageous, and rather the reverse with isolate 2SA. Isolates I and 18/M2 which grew well without dextran production in simple phosphate media, produced dextran in similar media containing a high concentration (1.5 %, w/v) of tryptose either dialysed or undialysed, provided that pantothenate was also supplied. Further trials showed that a smaller concentration of tryptose (0.3–1.2 %, w/v) had little effect in inducing dextran formation. No essential change in the properties of the strain had occurred in those instances where good growth without dextran production took place, since plating on to 3 % (w/v) sucrose + 3 % (w/v) Bacto-proteose peptone agar and subsequent incubation in 100 % CO₂ gave the usual large mucoid colonies typical of *S. bovis* under these conditions (cf. Bailey & Oxford, 1958*a*).

Table 1. *Effect of buffering and addition of pantothenic acid on growth and dextran production by five isolates of Streptococcus bovis*

The basal medium contained sucrose (5%), 'technical' Casamino acids (2.5%), and biotin, riboflavin, nicotinic acid, pyridoxine, p-amino-benzoic acid, thiamine and folic acid: incubation for 2 days at 37°.
Additions: P = calcium pantothenate; T = Bacto-tryptose (1.5% w/v)

Isolate no.	Property observed	P only	P + Acetate	Acetate only	P + phosphate	Phosphate only	T only	Dialysed T + P	T + P + phosphate	Dialysed T + P + phosphate
I	Growth	+	+	+	+	+	+	+	+	+
	Dextran	0	+	0	0	0	0	0	+	+
18/M2	Growth	-	+	+	+	+	+	+	+	+
	Dextran	-	+	0	0	0	0	0	+	+
2B	Growth	-	+	+	+	+	-	-	-	-
	Dextran	-	+	0	0	0	-	-	-	-
40	Growth	-	+	± (1 day) + (2 days)	+	+	-	-	-	-
	Dextran	-	+	0 (1 day) + (2 days)	0 (1 day) + (2 days)	0 (1 day) + (2 days)	-	-	-	-
2SA	Growth	-	+	+	±	+	-	-	-	-
	Dextran	-	+	0*	0	0 (1 day) + (2 days)	-	-	-	-

* Dextran = + + in second 'acetate only' culture inoculated from this culture.

Niven *et al.* (1948) found that their strains of *Streptococcus bovis* would grow in a simple sucrose + phosphate medium containing thiamine, pantothenate, nicotinic acid, biotin, arginine and glutamic acid. None of the five isolates of *S. bovis* used in the present investigation grew in this medium. Unlike ours, all the strains of Niven *et al.* fermented mannitol and in this and other respects were atypical, although apparently they could produce dextran.

DISCUSSION

The fact that rumen strains of *Streptococcus bovis* seem always to require biotin, and often pantothenate and perhaps other B-vitamins as well, for good anaerobic production of dextran from sucrose, yet will grow well anaerobically without any B-vitamins in a glucose medium (Ford *et al.* 1958) is another instance of the dependence of microbial B-vitamin requirements upon the task set the micro-organism (see also observations on *Leuconostoc* below). There is no evidence, however, to suggest that either of these vitamins is directly concerned in the production of the enzyme dextranase; it would seem rather that this enzyme can be produced in quantity only when growth of the organism is rapid and profuse, and this does not occur in sucrose media without the addition of B-vitamins. In this connexion it is instructive to compare the vitamin requirements of *S. bovis* with those of *Leuconostoc dextranicum* as reported by Whiteside-Carlson & Rosano (1951). The last-named organism is more exacting, since for full growth in sucrose media it required five B-vitamins, namely, nicotinic acid, thiamine, pantothenic acid, folic acid (or *p*-aminobenzoic acid) and either pyridoxal or pyridoxamine. Strangely, *L. dextranicum* does not require added biotin for growth or dextran production in sucrose media, although it does require added biotin for growth in glucose media. It differs, however, from *S. bovis* in not having a CO₂ requirement for dextran production (see Bailey & Oxford, 1958*b*).

In view of the earlier work of Bailey & Oxford (1958*a*) who grew *Streptococcus bovis* for dextran production in a phosphate-buffered medium (containing also yeast extract and Casitone), the finding that this organism can grow well in a simple sucrose + amino acid + phosphate medium of high buffer capacity in presence of ample CO₂ and yet produce no dextran was very surprising. The explanation for this behaviour is probably that, unlike acetate, phosphate does not act as a buffer in the narrow pH range over which dextranase is secreted into the culture, which is always tending to become more acid as a result of lactic-acid production. According to Dr R. W. Bailey (to be published) this secretion of enzyme takes place best at pH 5.5–6.0. The function of the soluble protein (non-dialysable) fraction of Bacto-tryptose in stimulating dextran synthesis in a phosphate-buffered medium is not yet fully understood. It may merely help to stabilize what little enzyme is secreted under these conditions. There can be no doubt that acetate is the buffer of choice in growing *S. bovis* for dextran production: it has hardly ever been observed to fail in presence of CO₂ and the appropriate B-vitamins.

Since it is all too easy to grow known dextran-synthesizing strains of *Strepto-*

coccus bovis in a sucrose medium and observe no dextran production, it is pertinent to inquire whether dextran formation is commoner among streptococci, especially of the viridans group, than the literature might lead one to suppose. The following statements are culled from the latest (7th) edition of *Bergey's Manual* (1957).

Viridans group. *Streptococcus salivarius* produces both levan and dextran from sucrose. *S. bovis*: 'some strains synthesize a dextran'. *S. mitis*: 'only rare strains produce large mucoid colonies on 5% sucrose agar'. *S. equinus*, *S. thermophilus* and *S. uberis*: 'no polysaccharide synthesized from sucrose'. Since the work of Dain, Neal & Seeley (1956) and of Bailey & Oxford (1958*a*) has shown that the above comment on *S. bovis* is an understatement, it might be worthwhile to re-investigate the last four viridans streptococci mentioned, with the use of techniques of buffering, vitamin addition and CO₂ incubation as in this present investigation. Furthermore, there may be hitherto unsuspected cases of possible dextran production among many groups of streptococci and related bacteria since *S. sanguis* (Group H) is known to produce dextran.

I am indebted to Dr R. W. Bailey for preparing the dialysed tryptose and also for many helpful discussions.

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The Effect of Residual Water on the Survival of Dried Bacteria During Storage

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SUMMARY: The optimum water activity (a_w) for survival of dried bacteria has been determined by storing the organisms in water-vapour equilibrium with solutions of known a_w value. The position of the optimum differed according to the composition of the suspending fluid, and the presence or absence of air in the storage atmosphere. For *Staphylococcus aureus* and *Pseudomonas fluorescens*, dried in papain digest and stored *in vacuo*, survival at 0.07, 0.11 and 0.16 a_w was somewhat better than at 0.00 a_w and 0.22 a_w . Survival at 0.33-0.53 a_w was much less. For *Salmonella newport* dried in the same medium the optimum a_w *in vacuo* was less clearly defined, but was well marked and close to 0.2 when stored in air. *S. newport*, when stored *in vacuo*, survived best at 0.22 a_w after drying in a salts buffer or in dialysed horse serum, but in whole serum or the dialysable serum fraction survival was better at 0.00 a_w . *S. newport* dried from water survived best at 0.00 a_w *in vacuo*, but at 0.43 a_w in air. In the presence of sucrose the effect of a_w was relatively small. In the presence of glucose or arabinose survival at 0.00 a_w was better than at 0.22 a_w , which in turn was much better than at 0.43 a_w . In the presence of all three sugars the differences between results *in vacuo* and in air were small.

Although it has been known for some time that insufficient drying, or excessive absorption of atmospheric moisture, causes a rapid decrease in the viability of dried bacterial cultures, there is no satisfactory evidence regarding the most suitable degree of residual moisture. It is known from the work of Stamp (1947) and Rhodes (1950) that storage over phosphorus pentoxide is, at least under some conditions, reasonably satisfactory. The need for very low water contents during storage has been emphasized by Proom & Hemmons (1949), who have advocated a period of secondary drying to make the cultures as dry as possible before sealing the ampoules for storage. This view has been questioned by Fry & Greaves (1951), who have suggested that the beneficial effects of glucose may have resulted from its ability to retain small amounts of water necessary for survival. Although Fry & Greaves did not furnish direct evidence in support of this suggestion, Hutton, Hilmoe & Roberts (1951) were able to demonstrate increased survival of *Brucella abortus* following a limited resorption of water vapour by the dried cultures. The amount of resorbed water which gave greatest survival was, however, too small to permit its satisfactory determination by direct analysis.

In addition to the difficulty of determining small amounts of water analytically, there is a further limitation that values so obtained apply only to the particular solute mixture in which the organisms are suspended. For instance Hutton *et al.* (1951) used the suspending fluid of Naylor & Smith (1946) which has a solids content of some 35 mg./ml., an amount greatly in excess of the

dry weight of the bacteria suspended in it. In these circumstances the measured water content of the dried culture cannot be regarded as an estimate of the water content of the bacterial cells. These difficulties and uncertainties have been avoided by storing the cultures under constant known water-vapour tension; the present paper reports some of the results obtained.

METHODS

Cultures for drying were grown on the surface of brain heart agar, or in broth aerated by shaking, for about 20 hr. at 30°. The growth on agar was washed off with a papain digest broth prepared as described by Proom & Hemmons (1949), but from ox heart instead of horse muscle. The suspension so obtained was, when necessary, divided into samples equal in number to the number of suspending fluids to be used, and centrifuged. The organisms were resuspended in the appropriate fluid to a density of about 10^{10} /ml. and 0.2 ml. samples dispensed in 88 × 9 mm. Pyrex test tubes. A small (20–25 mg.) cotton-wool plug was inserted *c.* 5 cm. from the mouth of each tube. The ampoules were supported in a rack so that each tube was radially mounted about 20° from horizontal, with its mouth some 2 cm. from the surface of the central condenser which was filled with solid CO₂ and ethanol. The apparatus accommodated up to 150 ampoules, and in most experiments 100–120 were dried together. Cooling was purely evaporative and heating was by radiation from the walls of the steel vacuum chamber. When desired, the temperatures of the contents of selected ampoules were measured during drying by means of forty-gauge thermocouples connected to a recording potentiometer. It was found convenient to de-gas the suspensions by evacuation until they had cooled to about 0–2° before the CO₂ + ethanol mixture was added to the condenser. Immediately the condenser was cooled the rate of evaporation increased, and organisms suspended in papain digest broth were promptly cooled from –30° to –35°. With this fluid almost all the drying occurred within the first hour, but drying was continued, nevertheless, for 4 or 5 hr. In the presence of some solutes drying took place at higher temperatures and from 3 to 4 hr. were needed to remove almost all the water.

At the end of this primary drying the ampoules were removed from the vacuum chamber, and, after inserting a small identifying label on top of the cotton-wool plug, each ampoule was placed inside a 150 × 15 mm. Pyrex tube for storage at a constant predetermined water activity (a_w). The a_w within the larger tube was controlled by placing within it *c.* 2 ml. of various aqueous solutions saturated at 25°, together with a small amount of the appropriate solid phase. For 0.07 a_w the solute was NaOH, and for this solution the glass surfaces likely to be exposed to it were first coated with a thin layer of solid paraffin.

The solutions used to generate the various values of a_w were from those listed by Robinson & Stokes (1955, Appendix 8. 11) with the addition of calcium bromide, a saturated solution of which was, at 25°, found to be in equilibrium with sulphuric acid of 0.162 a_w . For storage at zero a_w 1–2 g.

phosphorus pentoxide was placed in the bottom of the larger tube. Contents of the large tubes were protected against absorption of atmospheric moisture by inserting rubber stoppers until the tubes were sealed. For sealing, the larger tube was first drawn out to a neck and this was subsequently sealed with the aid of a cross-fire torch. Unless otherwise indicated the tubes were evacuated before sealing. This was done in groups of six on a manifold connected to a two-stage mechanical pump, the solutions at a_w 0.11 and above being boiled and the non-condensable gases thereby flushed out with the water vapour. Solutions of saturated NaOH did not boil easily, and these and the tubes containing P_2O_5 were pumped out for at least 60 sec. before the final sealing. When storage in air was desired the tubes were not evacuated before the final sealing. In any one experiment the time for sealing the requisite number of tubes never exceeded 3 hr. and was usually between $1\frac{1}{2}$ and 2 hr. Immediately after the completion of sealing, the tubes were stored in the dark inside an insulated cabinet within a room maintained at 25°. Temperature conditions within the cabinet were very steady, the daily fluctuations from the mean not exceeding 0.05°. The mean storage temperature was probably within the range $25.0 \pm 0.2^\circ$.

The general procedure has been to allot 5–12 replicate ampoules to each of several a_w values for storage and to withdraw single or duplicate ampoules after various storage periods for determination of the viable count. Precautions were taken to avoid bias when ampoules were allotted to the different a_w values for storage, and also when tubes were selected for withdrawal from storage.

Very few tubes developed cracks during storage, but care was taken to ensure that only tubes with intact seals were used for viable counts. Tubes were opened by first making a scratch with a tungsten carbide knife and leading a crack around the tube with the aid of a small (5 mm.) flame. This procedure, which has been used on several thousand tubes, caused a gentle release of the vacuum and rendered unnecessary other precautions against the violent ingress of air. After the outer tube was opened, the label and cotton-wool plug were removed from the small tube and the contents rehydrated with 2 ml. of saline. When necessary, further dilutions in saline were prepared, in which case the period of sojourn in the 1/10 dilution was not less than 90 sec. Duplicate plates were poured at appropriate decimal dilutions and the results are given as two figure logarithms of the viable count/ml. of original undried suspension. Owing to the exploratory nature of some of the experiments counts were sometimes appreciably above or below the expected values, and it was occasionally necessary to include estimates based on colony counts outside the range of 20–1000 colonies/plate.

RESULTS

Storage in papain digest in vacuo

As a papain digest similar to the one used by Proom & Hemmons (1949) had been adopted for routine preservation of dried cultures in this laboratory some experiments were designed to determine the optimum a_w value for

storage in this medium. Results for a strain of *Staphylococcus aureus* stored at eight a_w values from 0.00 to 0.53 are shown in Fig. 1. It is quite clear that, under these conditions, the driest environment did not result in the greatest survival.

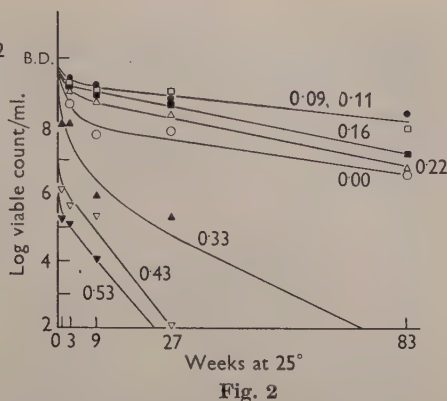
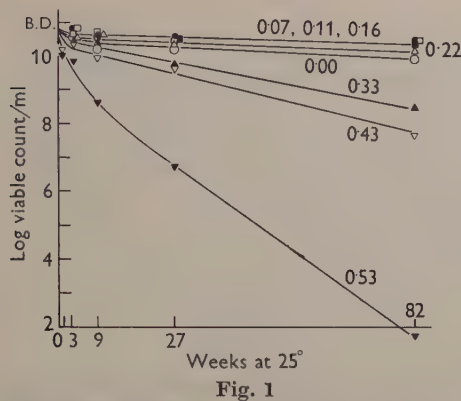


Fig. 1. Effect of a_w value on survival, during storage *in vacuo* at 25°, of *Staphylococcus aureus* dried in papain digest broth. Points are means of two estimates based on duplicate ampoules. (B.D. = before drying.)

Fig. 2. Effect of a_w value on survival, during storage *in vacuo* at 25°, of *Pseudomonas fluorescens* dried in papain digest broth. Points are means of two estimates based on duplicate ampoules.

Results for a second organism, *Pseudomonas fluorescens*, are shown in Fig. 2. This organism was dried in a portion of the same batch of papain digest as the *Staphylococcus aureus*, and has shown a greater rate of destruction when stored under the same conditions. Again, the driest conditions did not result in the best survival. Results for *Salmonella newport*, also dried in the same batch of papain digest, are shown in Fig. 3. For this organism the rate of death showed little variation at several a_w values between 0.00 and 0.22, although survival was markedly less at higher a_w values.

The water sorption isotherms at 25° for suspensions of the above three organisms in papain digest are shown in Fig. 4. These isotherms are for suspensions similar to those used for the experiments in Figs. 1, 2 and 3, and were obtained by the method described previously (Scott, 1953). The total dry matter was *c.* 50 mg./ml. of which *c.* 75 % was from the papain digest itself and 25 % from the organisms. It is clear, for the three suspensions studied, that the equilibrium water contents at the a_w values which gave greatest survival were some 3–4 % of the dry weight.

Storage in papain digest in air

The results of an experiment in which storage *in vacuo* and in air were compared at six a_w values are shown in Fig. 5. The test organism was *Salmonella newport*. The results for storage *in vacuo* were very similar to those shown in Fig. 3 for another batch of papain digest medium, dried some months earlier. The replicate tubes stored in air showed a very different response.

Whereas *in vacuo* there was little difference between storage at 0.00, 0.11 and 0.22 a_w , storage in air for 9 or 28 weeks caused much greater mortality at 0.00 a_w than at several other a_w values from 0.11 to 0.43.

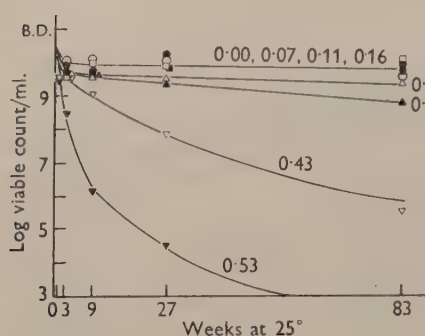


Fig. 3

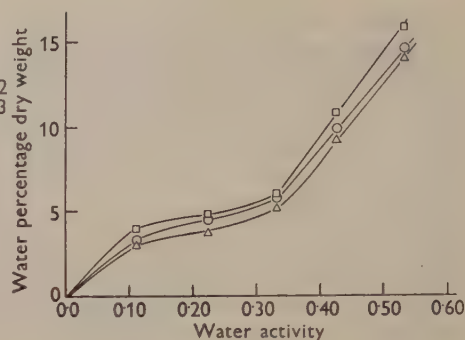


Fig. 4

Fig. 3. Effect of a_w value on survival, during storage *in vacuo* at 25°, of *Salmonella newport* dried in papain digest broth. Points are means of two estimates based on duplicate ampoules.

Fig. 4. Water sorption isotherms at 25° for three bacteria suspended in papain digest broth. □, *Staphylococcus aureus* 309; ○, *Salmonella newport* 215; △, *Pseudomonas fluorescens* 429.

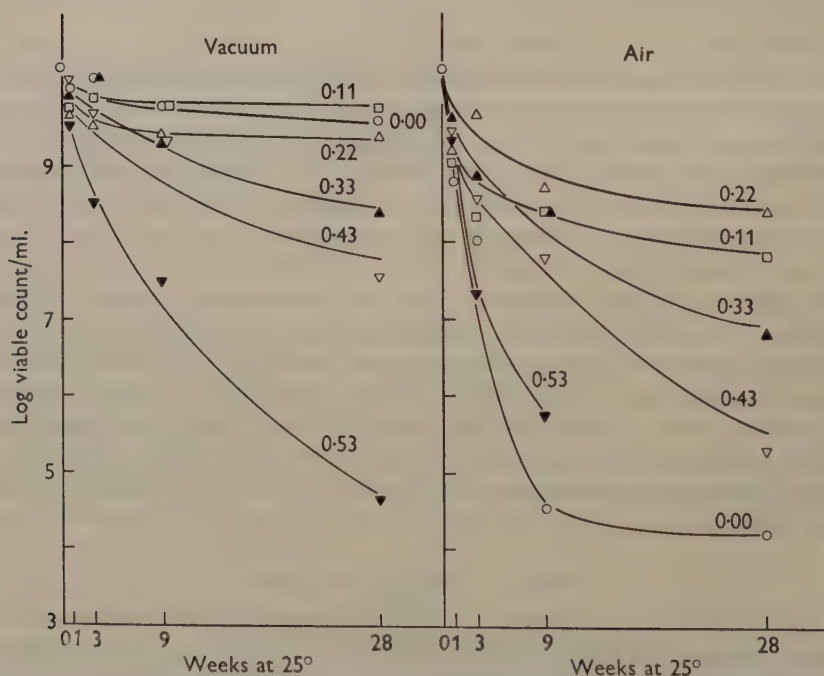


Fig. 5. Effect of a_w value on survival, during storage at 25° *in vacuo* and in air, of *Salmonella newport* dried in papain digest broth. Estimates of viable counts based on single tubes only.

Other experiments have shown that *Pseudomonas fluorescens* is similarly much more susceptible to very dry conditions when dried in papain digest and stored in air as compared with storage at the same a_w value *in vacuo*.

Storage in serum and serum fractions

Serum is widely recognized as containing substances which protect dried organisms, and numerous authors have ascribed this to 'protective colloids' (Fry, 1954). The evidence that the protective substances are colloidal is not clear, and does not appear to rest on comparative tests of the dialysable and non-dialysable fractions of serum. Table 1 summarizes the result of an experi-

Table 1. *Effect of a_w value on survival, during storage in vacuo at 25°, of Salmonella newport dried in various serum fractions*

Estimates of viable counts based on single tubes only.

Suspending fluid	Log viable count/ml.		Storage period (days)	Log viable count/ml. (storage a_w value)		
	Before drying	After drying		0.00	0.22	0.43
Salts buffer	10.55	8.64	8	5.51	5.48	4.63
			22	4.65	4.32	4.23
			85	4.60	4.27	3.65
			708	1.70	3.62	—
Horse serum	10.28	9.31	8	8.28	8.30	8.14
			22	8.23	7.58	7.75
			85	8.29	8.66	5.72
			708	7.77	6.18	—
Dialysable serum fraction	10.40	8.33	8	7.24	6.87	5.88
			22	7.09	6.84	4.64
			85	7.62	5.16	3.98
			708	7.64	4.90	—
Non-dialysable serum fraction	10.42	8.28	8	6.09	6.74	6.11
			22	4.72	5.93	5.33
			85	4.20	6.06	5.32
			708	1.70	4.61	—

ment in which the storage of *Salmonella newport* was examined at three a_w values in the presence of horse serum, and of its dialysable and non-dialysable fractions as well as in a simple salts buffer approximately isotonic with serum. After storage for 2 years there was greater death at 0.00 a_w than at 0.22 a_w in the presence of the salts and in the non-dialysable serum fraction. On the other hand, for the whole serum, and for the dialysable fraction, storage at 0.00 a_w gave the best survival. It may be seen also that these results have contradicted the proposition that the principal protective components of serum are colloidal.

Storage in the presence of sugars

In view of the suggestion by Fry & Greaves (1951) that the beneficial effects of glucose might depend on the retention of some significant amount of residual water some tests were made with bacteria dried in the presence of various sugars. The results of an experiment in which *Salmonella newport* was

dried in the presence of 1.1 molal sucrose, glucose and arabinose are shown in Fig. 6. In this particular experiment drying in the presence of each of these sugars was compared with drying in water only. For storage the ampoules were

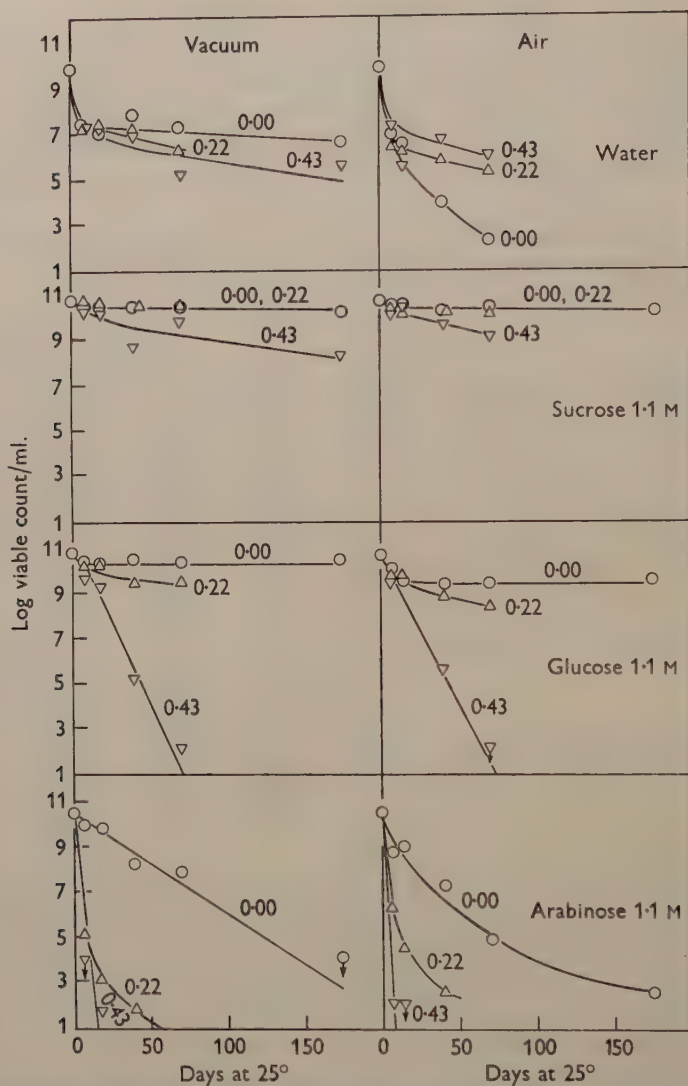


Fig. 6. Effect of a_w value on survival, during storage at 25° in *vacuo* and in air, of *Salmonella newport* dried in four different suspending fluids. Estimates of viable counts based on single tubes only.

taken in groups of four, one of each suspending fluid, and these were stored together by sealing within a larger 200 × 25 mm. tube. This procedure ensured that comparison of the four suspending fluids was not vitiated by minor differences in the storage conditions.

From an inspection of the results in Fig. 6 it may be concluded that for *Salmonella newport* the effect of residual water during storage depends markedly on the type of suspending fluid. For organisms suspended in water only, storage *in vacuo* has resulted in somewhat better survival at 0.00 a_w than at 0.22 and 0.43 a_w , but at each a_w value there was a large initial rate of death followed by a marked decrease to a much lower rate. For replicate ampoules stored in air the high initial rate of death was again followed by a low rate of death at 0.43 a_w , but at 0.00 a_w a relatively high rate of death continued beyond the first 2 weeks. For organisms suspended in water, therefore, the effect of air on the relationship between survival and a_w was similar to that found for organisms suspended in papain digest (cf. Fig. 4). For organisms suspended in sucrose death during storage was small at 0.00 and 0.22 a_w , and somewhat greater at 0.43 a_w . The high initial rate of death, which was so marked for organisms suspended in water, was no longer evident. The results for storage *in vacuo* and in air were similar. For cells suspended in glucose solution the changes in viability during storage depended very greatly on a_w . At 0.00 a_w storage *in vacuo* gave virtually the same high survival as in sucrose, although in air the mortality in glucose was somewhat greater. At 0.22 a_w mortality in the glucose was increased, and at 0.43 a_w a diminution of about 1 log unit/week was maintained. Although this steady rate was appreciably less than the high initial rate found in the suspension dried from water, it continued without appreciable change until sterility was approached. As a consequence the relative performance of glucose and water was a function of the duration of storage at 0.43 a_w . For example, in air at 0.43 a_w the viable count in glucose was some 200 times greater than the viable count in water after storage for only 7 days, whereas after 70 days the viable count for the water suspension was 10,000 times greater than in the glucose suspension.

For organisms suspended in arabinose the rate of destruction was much greater than in glucose at all three a_w . In arabinose death proceeded at a rate which was of the order of 0.25–0.5 log unit/week at 0.00 a_w and approached 1 log unit/day at 0.43 a_w .

DISCUSSION

The technique of storing the dried cultures over solutions of known constant a_w value undoubtedly caused the a_w value of the dried organisms to approach the equilibrium value very closely. The rate at which equilibrium was approached was, however, not determined and there is some uncertainty regarding the average a_w value of the organisms during the early part of storage. Observations on the papain digest suspensions suggested, however, that the greater part of the water taken up at 0.43 and 0.53 a_w was transferred in the first 24 hr. of storage. As at lower a_w values the amounts of water required to be transferred were much less, it is likely that conditions approaching equilibrium within *c.* 0.01 a_w were realized for most treatments within the first few days of storage. The approach to equilibrium over P_2O_5 or with the concentrated sugar solutions may conceivably have been much slower, but it seems unlikely that equilibration has been slow enough to affect the general conclusions.

The results provide clear evidence about the importance of residual water during storage, without requiring any dependence on analytical determinations of the water present. It is, moreover, likely that the a_w value would be a more satisfactory measure of the chemical reactivity of water in dried biological preparations than would estimates of water contents (Scott, 1957). Although the facts are clear enough, their interpretation is by no means simple. The reason for the accelerated rate of death at very low a_w values is not obvious, but apparently the removal of the most firmly held water molecules results in some loss of stability especially in the presence of air. In the presence of high concentrations of some sugars the damaging effects of air at low a_w values are substantially prevented, but whether this is simply a consequence of the hydrophilic character of the sugar molecule is unknown. It is of interest to find that the optimum a_w of c. 0.2 for the survival in air of *Salmonella newport* dried in papain digest is the same as the value reported by Watts (1945) for the survival in air of *Streptococcus agalactiae* dried in milk. On the other hand, Bullock & Lightbown (1947) found, with *Bacterium lactis aerogenes*, no difference in survival at 0.00 and at 0.32 a_w . Storage over P_2O_5 was *in vacuo*, but at 0.32 a_w the atmosphere was not specified. As they did not store material at any other value of a_w their results are not necessarily in conflict with those in the present paper.

The large differences between survival in sucrose, glucose and arabinose which are also a function of a_w value have been confirmed in several other experiments. These results suggest that the reducing groups of the latter two sugars might be a significant cause of death during the storage of dried bacteria. Evidence in support of this view has been presented elsewhere (Scott 1958).

The author is greatly indebted to Miss Betty J. Marshall whose skill and care produced the viable counts, and to Mr D. F. Ohye whose attention ensured the consistent performance of the drying apparatus.

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Lysogeny and Conversion in Mitis and Mitis-Like *Corynebacterium diphtheriae*

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SUMMARY: Fifty-six toxigenic mitis and mitis-like *Corynebacterium diphtheriae* strains isolated in the western part of the United States and Canada were examined for lysogeny and for the relationship of their phages to conversion to toxigenicity. Twenty-five strains (46 %) were demonstrably lysogenic. Twenty-two of the phages from the toxigenic strains were able to convert a sensitive non-toxigenic strain to toxigenicity. Due to technical limitations three phages could not be tested in a similar manner. On the basis of serological relationships, cross-immunizing ability and host range all 22 converting phages proved to be very closely related. They were also related to β - and γ -phages previously studied in connexion with the phenomenon of conversion to toxigenicity by bacteriophage.

Freeman's discovery (1951) that non-toxigenic *Corynebacterium diphtheriae* were converted to toxigenicity by bacteriophage was recognized immediately as an observation of great importance to a broadened understanding of the epidemiology of diphtheria. In spite of this, little information has been published relating directly to the natural occurrence or significance of the phenomenon he described. In the present study some of the questions which have been raised in relation to epidemiology have been investigated. A group of toxigenic mitis and mitis-like *C. diphtheriae* isolated in western United States and Canada were studied with the following questions in mind. How many of the isolates are lysogenic? How many of the lysogenic strains carry a phage capable of converting a sensitive non-toxigenic strain to toxigenicity? Are the phages carried by these strains related to each other and/or to the β - and γ '-converting phages (Groman & Eaton, 1955) previously studied in association with mitis-like *C. diphtheriae*?

MATERIALS AND METHODS

Bacteriophage and bacterial strains. Lyophilized cultures from the stock collection of *Corynebacterium diphtheriae* strains at the University of Washington were used. The cultures had been collected by Dr V. J. Freeman. Stock cultures of these strains were maintained on slopes of trypticase soy medium (Baltimore Biol. Labs.), supplemented with 1 % yeast extract and were stored at 4°. Transfers were generally made every 5-6 weeks. A list of the strains arranged according to their geographical origin is given in Table 1. Information was not available concerning the origin of each culture; therefore, it is possible that some of these cultures represent multiple isolates from the same individual. A number of other bacterial strains, C4, C4Sr (a streptomycin

resistant mutant of C4), C4/ β , C4(β), C4(γ), C7 and C1180 were employed as phage indicators during this investigation and represent strains which have been studied in relation to the conversion phenomenon. These strains were maintained on Loeffler's medium.

Table 1. *Strains of toxigenic Corynebacterium diphtheriae examined in this study*

A—Seattle, Washington		S—Washington State		V—Vancouver, British Columbia	
A 003	A030	S006	S040	V 327	V 359
A 004	A031	S036	S041	V 328	V 467
A 005	A032	S038	S047	V 339	
A 021	A035	S039		V 341	V 492
A 022	A036				
A 023	A037				
A 028	A045				
C—California		P—Portland, Oregon			
C 43	C1102	P17883	P18132	P 20490	
C 224	C1609	P17886	P18314	P 20491	
C 626	C1898	P17895	P18316	P 20492	
C 796	C1899	P17927	P18433	P 21804	
C 834		P17930	P18881	P 22840	
		P18009	P19099		
		P18011	P20488		

In addition to the phages isolated during this study, certain phages previously studied in this laboratory were employed. Phages β and γ are related temperate phages; β is able to convert sensitive non-toxicogenic strains of *Corynebacterium diphtheriae* to toxigenicity, while γ cannot. Phages β' and γ' are recombinants of β and γ . Phage B is a virulent mutant of β ; phage Bh is a host range mutant of B. The critical relationships among stock phages, stock strains, and the toxigenicity of stock strains are given in Table 2.

Table 2. *The interrelationships of Corynebacterium diphtheriae strains and phages*

Strains	Sensitivity to phage				Tests for lysogeny on indicator strains			Toxi-genicity
	β and β'	γ and γ'	B	Bh	C4(β) and C4(β')	C4/ β	C4(γ) and C4(γ')	
C4	+	+	+	+	—	—	—	—
C4(γ)	+	—	+	+	+	+	—	—
C4(γ')	+	—	+	+	+	+	—	+
C4(β)	—	+	—	+	—	—	+	+
C4(β')	—	+	—	+	—	—	+	—
C4/ β	—	+	+	+	—	—	—	—

Bacteriological methods. Difco heart-infusion broth (HIB) and a Proteose peptone salt (PPS) medium were used throughout the work for the propagation of the bacterial strains and bacteriophage. The heart-infusion medium was used exclusively for dilution and for plaque counting and had a final

pH of 7.2-7.4. The PPS medium, which consisted of 2% Proteose peptone (Difco) and 0.5% sodium chloride at a final pH of 7.8, was also employed in the '*in vitro*' test for toxigenicity. For this test the salt concentration was reduced to 0.25% and the medium was supplemented with 20% sheep serum. Toxin production was also determined by the intracutaneous method in guinea-pigs.

Both HIB and Neill's broth base medium were used in determining sugar fermentations. Neill's broth base consisted of 1% beef extract, 1% Proteose peptone no. 3 (Difco), and 0.5% NaCl and had a final pH of 7.3. Bromocresol purple was used as the indicator. Sucrose and glucose were incorporated into the base medium prior to sterilization, while maltose, starch and glycogen were added aseptically subsequent to sterilization. Final sugar concentrations were those recommended in Diagnostic Procedures and Reagents (1950). Inocula consisted of 0.1 ml. of overnight cultures grown in the particular base medium to be used. Reactions were recorded at 24, 48 and 72 hr. and at 1 week.

To test for haemolysin production, 1.0 ml. of a 48 hr. HIB culture of the bacterium was added to 1.0 ml. of a 2% suspension of fresh human red blood cells which had been washed three times in 0.01 M-phosphate buffered saline, pH 7.0. The tubes were placed in a 37° water bath for 1 hr., then refrigerated overnight. Haemolysis was read as present or absent. A control tube of sterile broth plus the red blood cells was included and was always negative.

Nitrate reduction was tested by incorporating 0.1% KNO₃ into HIB. One-tenth ml. of an overnight HIB culture was used as the inoculum. Reduction to nitrite was tested at 48 hr. Cultures negative at this time were retested at the end of a 1 week incubation period, and those still negative were tested for the presence of nitrate by adding a few grains of powdered zinc to the medium following the addition of 0.5 ml. each of 0.8% sulphanilic acid and 0.5% alphanaphthylamine.

Christensen's urea agar slants (1946) adjusted to pH 7.2, were used to test for urease production. The slants were streaked and stabbed. Reactions were checked at 24, 48 and 72 hr. and at 1 week.

Mueller's serum tellurite medium (Mueller & Miller, 1946) was used to determine colonial morphology.

Bacteriophage technique. Plaque counts, tests for phage sensitivity and for lysogeny, and preparation of stocks of phages B, Bh, β and γ were carried out as previously outlined (Groman & Eaton, 1955). Stocks of phages from newly identified lysogenic strains were prepared in a manner similar to that described by Parsons (1955).

The isolation of lysogenized C4Sr strains. In the course of the present work strain C4Sr was lysogenized with 22 phages derived from newly identified lysogenic strains. C4Sr was employed to provide a marker (streptomycin resistance) which would distinguish the recipient strain from the phage donor strains, which were all streptomycin sensitive. In each case strain C4Sr was exposed to the phage either in broth or in an agar overlay, and following lysis and regrowth the culture or material from a plaque was streaked out on heart-

infusion agar. Well-isolated clones were then tested for phage production. One productive clone was restreaked and five well-isolated subclones retested for phage production. When necessary, the re-isolation process was continued until all subclones produced phage. At this time a subclone was transferred to stock as a lysogenic derivative of strain C4Sr. All cultures were checked and found resistant to streptomycin; and in addition, all were shown to agglutinate with an antiserum to C4Sr. The latter test provided a double check on the relationship of most of the lysogenic strains to the original C4Sr strain, since 19 of the 22 donor strains were antigenically unrelated to C4Sr.

RESULTS

Characterization of the strains. In selecting strains of *Corynebacterium diphtheriae* for study, those catalogued as mitis type were chosen. Since there were no experimental records of the criteria employed in typing these strains a redetermination of type was undertaken along with certain other biochemical tests.

All the strains which are listed in Table 1 produced acid from glucose and maltose, did not ferment sucrose, glycogen or starch, did not produce urease and with the exception of SO 47 reduced nitrate to nitrite. None produced a pellicle when grown in broth. All the strains listed were toxigenic. Their cellular morphology was in agreement with that expected for *Corynebacterium diphtheriae*. Haemolysin production and colonial morphology were more variable among these strains. Six strains (V 327, V 328, V 329, V 341, P 18314, P 20490, failed to produce haemolysin and 8 strains (P 17895, P 17930, P 18011, P 18132, P 18316, P 19099, P 20491, P 21804) gave both positive and negative results in a series of three tests.

The typical colonial morphology of mitis strains described by Mueller & Miller (1946) on serum tellurite medium, i.e. colonies 1-1.5 mm., black, convex and glistening, was exhibited by only a few of the strains tested. The majority were atypical in that their colonies exhibited black centres with a grey-white edge or black centres with a lighter concentric ring and an outer white edge. A few had colonies with grey or brown centres with a white edge. In spite of these differences the large size, convex contour, glistening appearance, and black or dark centres clearly qualified these colonies as mitis or mitis-like rather than gravis or intermedius. On the basis of all these observations the 56 strains studied in this investigation were classified as mitis or mitis-like *Corynebacterium diphtheriae*.

The extent of lysogeny. Two methods were employed in testing for lysogeny. In the first method supernatant fluids of the cultures to be tested were pooled in groups of three. Each culture was grown to approximately 4×10^8 cell/ml. and centrifuged for 20 min. at 3000 rev./min.; equal samples of the supernatants were pooled. A loopful of each pool was spotted on each of the 56 strains being studied and on stock strains C4, C7 and C1180. When employed as an indicator, each strain was incorporated into an agar overlay as for plaque counting, two plates per indicator strain sufficing for the nineteen pool tests.

When a given pool exhibited activity, the strains contributing to the pool were then tested individually against the sensitive indicator to determine the phage carrier(s).

In the second method employed a loopful of the supernatant fluid of each culture was spotted on stock strains C4 and C7. These indicators were selected on the basis of their known sensitivity to stock *Corynebacterium* phages. On the basis of the pool tests it was apparent that strain A028 displayed a wider range and a greater sensitivity to phage than our stock strains, and each toxigenic strain was tested individually on it as well. One other stock strain, C1180, was also sensitive to a number of phages. However, not all strains were tested on it individually as it appeared to be sensitive to the phages active on C4 and C7 and in fact was a second isolate from the same diphtheria patient yielding C7 (Freeman, 1951).

The results of tests with all identifiable lysogenic strains and three non-lysogenic strains are given in Table 3. The host range of the phages carried by the lysogenic strains is given in columns 2 to 8. The indicator strains

Table 3. *The sensitivity of Corynebacterium diphtheriae strains to stock phage and the host range of their carried phages*

Strains	Tests for lysogeny on indicator strains							Sensitivity to phage			
	A028	C1180	C4	C7	C4(β)	C4/ β	C4(γ)	γ	β	B	Bh
A023	+	+	+	+	+	+	-	-	+	+	+
C1899	+	+	+	+	+	+	-	-	+	+	+
C1102	+	+	+	+	+	+	-	-	+	+	-
C43	+	+	+	+	+	+	-	-	-	+	+
C1898	+	+	+	+	+	-	-	-	-	+	+
A003	+	+	+	+	+	+	-	-	-	+	+
A004	+	+	+	+	+	+	-	-	-	+	+
A005	+	+	+	+	+	+	-	-	-	+	+
A022	+	+	+	+	+	+	-	-	-	+	+
S006	+	+	+	+	+	+	-	-	-	+	+
S036	+	+	+	+	+	+	-	-	-	+	+
S038	+	+	+	+	+	+	-	-	-	+	+
S039	+	+	+	+	+	+	-	-	-	+	+
S040	+	+	+	+	+	+	-	-	-	+	+
C224	+	+	+	+	+	+	-	-	-	+	-
V467	+	+	+	+	+	+	-	-	-	+	-
C796	+	+	+	+	+	+	-	-	-	-	-
C834	+	+	+	+	+	+	-	-	-	-	-
C1609	+	+	+	+	+	+	-	-	-	-	-
A030	+	+	+	+	+	+	-	-	-	-	-
A031	+	+	+	+	+	+	-	-	-	-	-
A037	+	+	+	+	+	+	-	-	-	-	-
P17886	+	+	+	-	-	-	-	-	-	-	-
P18011	+	-	-	-	-	-	-	-	-	-	-
P21804	+	-	-	-	-	-	-	-	-	-	-
V359*	-	-	-	-	-	-	-	-	+	+	-
A045	-	-	-	-	-	-	-	-	+	+	+
A028	-	-	-	-	-	-	-	+	+	+	+

* This group of three contains the only non-lysogenic organisms exhibiting sensitivity to stock phages.

listed are all those on which a positive test was obtained. Strain C4(γ) was included for purposes of characterizing the host range pattern of the phages. The data show that 25 of the 56 cultures tested, or 46 %, were lysogenic. The maximum number of lysogenic strains was detected by A028, although the sensitivity of strains C4, C7 and C1180 was almost as good. It is clear that 46 % represents a minimal estimate of lysogeny in this group of cultures, since tests with additional indicator strains or the employment of more sensitive methods might well add to the number of positives.

The host range exhibited by the phages carried by these strains was identical, with the exception of the phages carried by strains C1898, P17886, P18011, and P21804. It is worth noting that only one of the 56 toxigenic strains tested, A028, was sensitive to any of the newly isolated phages as determined by the pool tests. Nontoxigenic indicator strains C4, C7 and C1180 were also screened for lysogeny in the pool test, but no phage activity was noted.

In addition to checking all 56 strains for lysogeny, each strain was tested for its sensitivity to stock phages (Table 2, columns 9–12). The strains have been arranged in groups which reflect their pattern of phage sensitivity. Of the 56 strains tested, 19 (34 %) were sensitive to one or more stock phages. Sixteen of the 19 sensitive strains were members of the lysogenic group and only 3 (the last group in the table) of the 31 non-lysogenic strains were sensitive. These patterns of phage sensitivity do not correlate with the general geographical origin of the cultures although more detailed knowledge of their origin might reveal some intra-group correlations.

The relationship of the newly isolated phages to β - and γ -phages. The relationship between the phages isolated from the lysogenic strains and β - and γ -phages, which are themselves closely related (Groman & Eaton, 1955), was determined next. As indicated above, with but four exceptions, the phages carried by the lysogenic strains exhibited an identical host range on all available indicator strains. However, host range is not a good criterion for demonstrating relationships among phages. Two criteria which are acceptable are serological relationship and the ability of temperate phages to immunize a given host to attack by the same phage and in most cases to closely related phages.

In order to determine whether the new phages were related to β and γ each phage was exposed to an antiserum produced against γ -phage which neutralized both β and γ at the same rate. Stocks of all but 3 of the unknown phages were prepared by growth on either C4 or A028. Ideally the inactivation rate constant of each phage should have been determined over a series of time intervals as a measure of its relationship to β and γ . However, this approach was technically not feasible and as a quantitative measure of relatedness the percentage inactivation of the unknown phage was compared with that of β -phage over a single time interval.

The results of these experiments, which are given in Table 4, clearly show that on the basis of the serological criterion all the phages isolated from the lysogenic strains are closely related to β and γ and inferentially to each other. The phages carried by the three lysogenic P strains were not included in these tests.

Table 4. *The rate of inactivation of the newly isolated phages and β -phage by β -phage antiserum*

Phage donor strain	% inactivation*		Phage donor strain	% inactivation*	
	Phage from donor	β -phage		Phage from donor	β -phage
C43	40	45	A022	49	31
C224	57	44	A023	42	39
C796	69	31	A030	51	40
C834	47	39	A031	51	31
C1102	73	45	A037	55	40
C1609	72	40	S006	62	44
C1898	65	31	S036	74	46
C1899	46	45	S038	36	45
A003	67	58	S039	76	58
A004	55	45	S040	60	31
A005	65	33	V467	41	44

* All phages were incubated for 10 min. at 37° with a 1:20 dilution of antiserum. β -phage inactivation was determined simultaneously with that of the unknown phage. Normal serum controls were included in each experiment. The 10 min. period was selected after preliminary experiments had indicated that inactivation was still progressing logarithmically at this time.

In order to carry out a significant test for the cross-immunizing ability of the newly isolated phages it was necessary to lysogenize a common bacterial host with each of these phages and isolate the lysogenic derivatives. Strain C4 was selected as the appropriate host since it was sensitive to 23 of the total of 25 phages. Since it was also non-toxigenic, the converting ability of these phages could be assessed simultaneously. The methods employed in the isolation of these lysogenic strains were outlined in the Material and Methods section, and it will be recalled that a streptomycin resistant mutant of C4, i.e. C4Sr, was employed as the recipient host.

Lysogenic C4Sr derivatives of 22 of the 23 phages active on this strain were isolated. Numerous efforts were made to obtain a strain lysogenized with the phage from P17886 but all failed. Each of the newly isolated strains, which will be designated C4Sr(X) strains, where X represents the strain designation of the phage donor, was tested for sensitivity to the phage carried by each of the other C4Sr(X) strains. In addition, the phage carried by each C4Sr(X) strain was tested for its host range on a group of selected indicators. Finally, each C4Sr(X) strain was tested for its sensitivity to stock phages β , γ , B and Bh. A summary of the findings is given in Table 5.

The data clearly show that each of the phages isolated during this study immunizes a common host to attack by all the others. Coupled with the serological relationship this finding established the relation of these phages to β - and γ -phage and to each other. With three exceptions this common immunizing property extends to immunizing C4Sr to stock phages as well. The added resistance of strains C4Sr(A003), C4Sr(S038) and C4Sr(V467) to β -phage might suggest that these phages are different in some degree from the others. However, we have observed (unpublished observations) that C4/ β

mutants, i.e. mutants resistant to β -phage, are very common in C4 cultures. It is quite likely that the three exceptions are in fact C4Sr(X)/ β strains and that the phages they carry are similar in all respects to the others.

Table 5. *The host range and immunizing ability of phages carried by the C4Sr(X) strains*

Bacterial strain	Phages from strains C4Sr(X)*	Sensitivity to				Toxi-genicity
		Stock phage				
		γ	β	B	Bh	
C4Sr(X)†	—	—	+	+	+	+
C4Sr(A003)	—	—	—	+	+	+
C4Sr(S038)	—	—	—	+	+	+
C4Sr(V467)	—	—	—	+	+	+
A028	+	+	+	+	+	+
C7	+	+	+	+	+	—
C4	+	+	+	+	+	—
C4(γ)	—	—	+	+	+	—
C4(β)	+	+	—	—	+	+
C4/ β	+	+	—	+	+	—

* X refers to phages obtained from each of the strains in the following list:

C43	C1609	A005	A031	S006
C224	C1898	A022	A037	S036
C796	C1899	A023	S038	S039
C834	A003	A030	S040	V467
C1102	A004			

† X refers to all strains in the above list less the 3 strains listed individually in the Table.

In addition to a common immunizing ability, all 22 phages had identical host ranges when tested against stock indicator strains (bottom portion of Table 5). On the basis of both of these characteristics it can be stated that all the phages are related to or identical with γ or γ' (see Table 2 also). It will be noted that the pattern of phage sensitivity of the C4Sr(X) derivatives differs in most instances from that exhibited by the parent strains supplying the phage (see Table 3). This is not unexpected, since the broadened resistance to phage infection exhibited by the parent strain may have developed independently, e.g. by mutation or as a result of lysogenization with other phages. It is this very fact which dictated the need for a common host in testing cross-immunizing ability among the phages isolated in this study. It is also worth noting that most of the parent strains also act as β -resistant strains in a manner analogous to C4Sr(A003), C4Sr(S038) and C4Sr(V467), indicating that this type of strain arises under natural conditions.

Converting ability of phages isolated from toxigenic strains. One of the primary purposes of this survey was to determine whether phages isolated from toxigenic strains were able to convert non-toxigenic strains to toxigenicity. As indicated in Table 5, the lysogenic C4Sr derivatives of 22 phages obtained from toxigenic strains were all toxigenic when tested by either the 'in vitro' plate or guinea-pig intracutaneous method. Thus each toxigenic strain which

could be tested was carrying a converting phage. The phage from one Oregon strain, P17886, was also tested for its converting ability on C4Sr. When cultures containing C4Sr and phage P17886 were streaked on 'in vitro' plates, positive tests for toxin production were consistently obtained, indicating the presence of a converting phage; but as mentioned earlier, a lysogenic C4Sr derivative could not be isolated.

Serological relationship of the toxigenic strains to C4. Another question studied was whether strains sensitive to the same phages as C4, or strains producing phage which attacked C4, were serologically related to C4. To test for such a relationship a slide agglutination test was used in which a C4 agglutinating antiserum was employed. The method of testing was that described by Minzel & Freeman (1950). Eight of the 56 toxigenic strains agglutinated with C4 antiserum. These were C626, C796, C834, C1609, V327, V328, V339 and V341. Of the 23 strains carrying phage active on C4, 3 were serologically related to C4 (C796, C834, C1609); these strains were from the same geographical area as C4. Although C4 is sensitive to β -phage, none of the 6 strains sensitive to β -phage agglutinated with C4 antiserum. On the basis of these results there is no recognizable relationship between phage production or sensitivity to phage and agglutination as determined by the present test.

DISCUSSION

There seems to be no question that lysogeny is a common property among the various types of *Corynebacterium diphtheriae* (Keogh, Simmons & Anderson, 1938; Thibaut & Frédéricq, 1952, 1956; Hewitt, 1952; Christensen, 1957). While we have found that 46 % of the toxigenic mitis or mitis-like strains were demonstrably lysogenic, the percentage of lysogeny reported by others among all types of *C. diphtheriae* has ranged from zero to values approximating those found in the current study. A true comparison of the extent of lysogeny among the various types of *C. diphtheriae* would be of theoretical interest; however, such a comparison is devoid of meaning, since in any study the detection of lysogeny depends on the fortuitous selection of appropriate indicator strains.

One of the most important questions raised by the conversion phenomenon is that of the role it plays in the natural evolution of toxigenic strains of *Corynebacterium diphtheriae*. One approach to this question is to examine toxigenic strains for the presence of converting phage. While lysogeny is a common characteristic of toxigenic *C. diphtheriae*, the presence or absence of converting phages in such strains has not been studied extensively. Hewitt, (1954) reported that 5 toxigenic gravis strains yielded converting phages, and Narbutowicz (1955) isolated a converting phage from a toxigenic mitis strain. In the present study converting phage was isolated from 22 toxigenic mitis or mitis-like strains and in fact was demonstrated in every instance where a reasonably convenient means for its demonstration existed. These findings strengthen the hypothesis that conversion is a major mechanism in the production of toxigenic *C. diphtheriae*. While the presence of a converting

phage in a toxigenic strain does not prove its role in the production of toxin, the inference is strong.

The finding that all of the converting phages isolated in the present study appear to be identical merits mention. One obvious explanation is that the indicator strains employed could only detect phages of this particular type. Nevertheless, the fact that 46 % of the strains tested were carrying this type of converting phage suggests that a fair proportion of the mitis strains present in the area of origin were carriers of this phage.

A final point with respect to the present findings is whether there is some significance to the observation that the converting phage type isolated from the western strains is related to the β - and γ -phages studied previously. β -phage was not isolated in Washington and probably was isolated in Ontario or Quebec, Canada, by Toshach from whom Freeman received it (Freeman, 1951). A phage apparently related to β has also been isolated by Narbutowicz in Poland (1955). It would thus appear that phages of this type are present in widely separated areas of the world. More studies are needed to determine whether there are other converting phage types as determined by the serological criterion or whether this is indeed a special class of phages. Ideally such studies should be carried out with converting phages isolated from all types of *Corynebacterium diphtheriae*.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its twenty-sixth General Meeting in the Royal Institution, London, on Monday, Tuesday and Wednesday, 14, 15 and 16 April 1958. The following communications were made:

COMMUNICATIONS

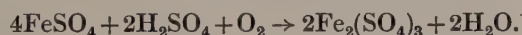
Isolation and Some Characteristics of an Iron-Oxidizing Bacterium. By

JAY V. BECK* (Department of Bacteriology, Brigham Young University, Utah) and S. R. ELSDEN, (Department of Microbiology, University of Sheffield)

A ferrous iron-oxidizing bacterium has been isolated from acidic water used in a copper recovery process in Brigham Canyon, Utah. Enrichment cultures grow well on a medium having the following composition in g. per litre: MgSO_4 , 0.2; KH_2PO_4 , 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20.0 and $\text{pH} = 2.5-3.0$. Pure cultures were obtained by streaking from an enrichment culture to a washed agar medium. The bacterium has been shown to grow better at 30° than at either 25° or 37° and to thrive at pH values as low as 2.0. Good staining results were obtained by heating with carbol erythrosin. The bacterium is a small, motile rod with a single polar flagellum. It is probably identical to *Ferrobacillus ferrooxidans* (Leathen & Braley, 1954).

Cells for Warburg studies were obtained by centrifugation of liquid cultures. The cell density in the stationary phase of a liquid culture is about 3×10^7 cells per ml. and cell suspensions from 1 l. of cultures made to 5 ml. contain 0.5–0.7 mg. cellular carbon per ml.

Oxygen consumption by cell suspensions in presence of ferrous ions at pH 2.6 and 30° was found to agree with the following equation:



Oxidation of ferrous ions by cell suspensions is independent of carbon dioxide absorption. However, if carbon dioxide is present it is fixed in amounts proportional to the iron oxidized.

The molecular ratio of carbon dioxide fixed to ferrous ion oxidized determined by use of radioactive carbon in Warburg experiments and growing cultures and by cellular carbon estimations on growing cultures varied from 0.0045 to 0.010. These values expressed as the molecular ratio of oxygen utilized to carbon dioxide fixed range from 25 to 55 and are similar to ratios reported for other chemolithoautotrophic bacteria. Results with growing cultures were consistently higher than with Warburg experiments.

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* Guggenheim Foundation Fellow, 1957–58.

The Critical Population for the Formation of Antibiotic Inhibition Zones in Staphylococcal Agar Cultures. By K. E. COOPER, A. H. LINTON and S. N. SEHGAL (*Department of Bacteriology, University of Bristol*)

The Action of Antrycide on Nucleic Acid Synthesis in a Trypanosomid Flagellate. By B. A. NEWTON (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Antrycide (2:6'-dimethyl-6:4'-pyrimidylamino-4:2'-diaminoquinoline-1:1'-dimethosulphate) has an outstanding activity against numerous trypanosoma species (Curd & Davey, 1950). Histochemical studies led Omerod (1951) to suggest that this compound might act by splitting cytoplasmic ribonucleo-protein into its constituent ribonucleic acid and protein. The present communication describes the effect of antrycide on the growth of a trypanosomid flagellate, *Strigomonas oncopelti*, and on the synthesis of nucleic acid by washed cell suspensions of this organism.

It has been found that the addition of antrycide (10 μ g. ml.) to cultures of *S. oncopelti* growing logarithmically in a peptone glucose medium, resulted in an immediate change from logarithmic to linear growth. This effect could be reversed by transferring organisms to a drug-free medium. Analysis of organisms harvested at intervals during linear growth in the presence of drug revealed that ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and protein were all synthesized at linear rates.

Conditions have been determined which permit the synthesis of nucleic acid and protein by washed cell suspensions of *S. oncopelti* (Newton, 1957). Antrycide had no effect on the net synthesis of RNA or DNA by washed cell suspensions for a period of about 4 hr. after the addition of drug, nor was the incorporation of ^{14}C -uracil or ^{14}C -glycine into the nucleic acid fraction of cells significantly affected. The incorporation of ^{14}C -adenine and ^{14}C -guanine, however, was 80-90% inhibited from the time drug was added; this inhibition was unaffected by the purine:drug ratio. These results suggest that, for the case of *S. oncopelti*, antrycide may act at some point in nucleic acid synthesis rather than by combining with and disorganising nucleic acid.

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Some Interactions Between Factors Affecting the Viability of Dried Micro-Organisms. By W. J. SCOTT (*Commonwealth Scientific and Industrial Research Organization, Division of Food Preservation and Transport, Homebush, N.S.W., Australia*)

Results of experiments reported elsewhere have supported the hypothesis that Maillard-type reactions, between carbonyl compounds and amino side-chains

of some essential cellular constituents are an important cause of death of dried micro-organisms. Further testing of this hypothesis with the aid of factorial experiments, has revealed a number of interactions between various solutes and environmental factors which influence the retention of viability. In a 3^4 experiment with *Salmonella newport* at three levels each of sucrose, glucose, ribose and of the water activity, five of the six possible first order interactions were significant after storage at 25° for 9 weeks. The effect of each of the sugars was influenced by the presence of the other two, and for the two reducing sugars the effect on viability was a marked function of the water activity. In another 3^4 experiment using *Pseudomonas fluorescens* stored at three levels each of sucrose, arabinose, glutamic acid and semicarbazide, only two of the first-order interactions were significant after 9 weeks, but some of the second-order interactions increased during storage. In a complete 2^6 factorial using a mixture of the above two bacteria, a considerable number of interactions appeared. The factors were sucrose, glutamic acid, semicarbazide, ribose, water activity and temperature, the first three being beneficial and the last three deleterious at the higher level employed. Analyses of variance of the log viable counts showed, for either or both organisms, that eleven, nine, and one of the first- second- and third-order interactions were statistically significant on one or more occasions during the first 27 weeks of storage. The magnitudes of all interactions changed with time, and sometimes differed between organisms. The magnitude and frequency of the interactions increase the difficulties in the planning, execution and interpretation of experiments on the storage of dried micro-organisms.

The Cell Wall of Chlorella. By D. H. NORTHCOTE (*Department of Biochemistry, University of Cambridge*)

The Metabolism of DNA in *Escherichia coli* infected with Bacteriophage T5. By L. V. CRAWFORD (*Department of Biochemistry, University of Cambridge*)

The infection of *Escherichia coli* B with phage T5 initiates a rapid decrease in the total deoxyribonucleic acid (DNA) content of the culture. Addition of chloramphenicol to the medium reduces the rate of DNA breakdown and the rate of protein synthesis to the same extent. Injection of the phage DNA into the host does not appear to be necessary for the DNA breakdown, as interference with injection by the addition of sodium citrate (Luria & Steiner, 1954) does not affect the rate or extent of the DNA breakdown. About 10 min. after infection the host DNA content is reduced to 30% of the initial amount. DNA synthesis then begins and soon reaches a high linear rate. The period of synthesis is terminated by the lysis of the cells. The addition of chloramphenicol during the period of DNA synthesis stops the increase of DNA within a few minutes of the addition. The lag in the effect of the drug is not due to a lag in the effect on protein synthesis. The inhibition of DNA synthesis by chloram-

phenicol is probably an indirect effect although no visible lysis accompanies the inhibition.

Although the DNA metabolism of phage T5-infected *E. coli* shows some unusual features, there are several similarities between the metabolism of phage T2-infected *E. coli* and phage T5-infected *E. coli*. These include the inhibition of net RNA synthesis by infection, the high rate of DNA synthesis as compared with that in the uninfected host, the activation of DNAase and the requirement for 'prior protein synthesis' before DNA synthesis. Also infection with phage T2, T4, T5, or T6 confers on *E. coli* B3, a thymine-requiring strain, the ability to make thymine. These similarities suggest an affinity between phage T5 and phage T2 in spite of the chemical difference between the nucleic acids of the two phages, phage T5 containing cytosine and phage T2 containing 5-hydroxymethyl cytosine (Wyatt & Cohen, 1953).

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 WYATT, G. R. & COHEN, S. S. (1953). *Biochem. J.* **55**, 774.

Followed by a Symposium on

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The Relation of Strategy to Tactics: Some General Biochemical Principles. By D. D. WOODS and R. G. TUCKER (*Microbiology Unit, Biochemistry Department, University of Oxford*)

The Antibiotic Approach. By H. B. WOODRUFF and L. E. McDANIEL (*Microbiological Research Division, Merck and Co., New Jersey*)

Selective Inhibition of Bacterial Cell Wall Synthesis; its Possible Applications in Chemotherapy. By J. T. PARK (*Walter Reed Army Institute of Research, Washington, D.C.*)

Surface Active Bactericides. By B. A. NEWTON (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Membrane Penetration and the Therapeutic Value of Chemicals. By P. D. MITCHELL (*Department of Zoology, University of Edinburgh*)

Inhibitors of Energy-Supplying Reactions. By H. A. KREBS (*Department of Biochemistry, University of Oxford*)

Metal-binding agents in Chemotherapy: the Activation of Metals by Chelation. By A. ALBERT (*Australian National University, Canberra*)

The Designing of Anti-Metabolites. By D. W. WOOLLEY (*Rockefeller Institute for Medical Research, New York*)

Lethal Synthesis. By R. MARKHAM (*Agriculture Research Council Virus Research Unit, Molteno Institute, University of Cambridge*)

- Selective Inhibition of Virus Multiplication.** By I. TAMM (*Rockefeller Institute for Medical Research, New York*)
- Specific Inhibitors of Protein Synthesis.** By E. F. GALE (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)
- Mechanisms of Chemotherapeutic Synergy.** By B. W. LACY (*Westminster Hospital Medical School, London*)
- The Chemotherapy of Bacterial Infections.** By R. KNOX (*Guy's Hospital Medical School, London*)
- The Chemotherapy of Fungal Diseases.** By R. J. W. BYRDE (*Research Station, Long Ashton, Bristol*) and G. C. AINSWORTH (*Commonwealth Mycological Institute, Kew*)
- The Chemotherapy of some Protozoal Infections; Factors Affecting the Chemotherapy of Amoebiasis.** By L. G. GOODWIN and R. A. NEAL (*Wellcome Laboratories of Tropical Medicine, London*)



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